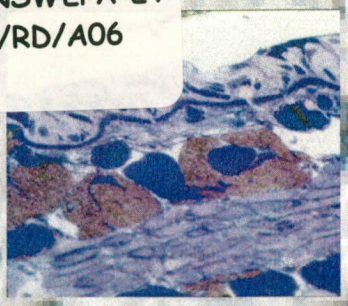

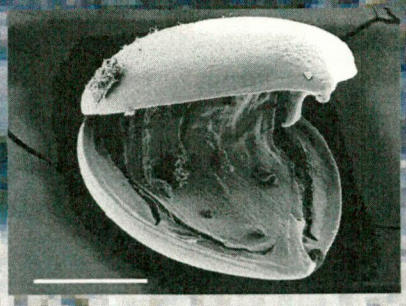

USE OF THE HISTOLOGICAL AND REPRODUCTIVE RESPONSES OF FRESHWATER MUSSELS AS BIOINDICATORS TO MONITOR THE HEALTH OF THE HAWKESBURY-NEPEAN RIVER.

Summary

FINAL REPORT: ENVIRONMENTAL RESEARCH TRUST
GRANT NO 94/RD/A06

MARIA BYRNE
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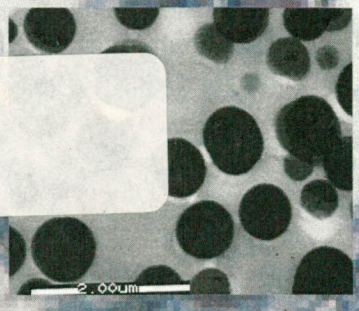

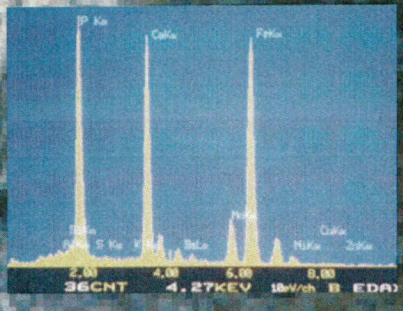

USE OF THE HISTOLOGICAL AND REPRODUCTIVE RESPONSES OF FRESHWATER MUSSELS AS BIOINDICATORS TO MONITOR THE HEALTH OF THE HAWKESBURY-NEPEAN RIVER.

SUMMARY

Final Project Report
Environmental Research Trust Grant no 94/RD/A06
October 1998

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PROJECT SUMMARY

This final project report presents a summary of a research program on reproduction and bioaccumulation in the freshwater mussel Hyridella depressa from the Hawkesbury-Nepean River system. The broad aims were to assess the effect of environmental degradation on reproduction in these ecologically important organisms and to assess their reproductive and element-accumulation responses as tools for monitoring environmental heath.

Reproduction and Conservation Biology of Hyridella depressa

Reproduction of six populations of H. depressa was examined over two breeding seasons. The aims of this study were to determine the reproductive condition of H. depressa from reference and impacted sites to assess the long-term impact of environmental degradation on recruitment. Reproduction in H. depressa is highly sensitive to aquatic trophic status and this was reflected in among site differences in reproductive output. At eutrophic sites influenced by anthropogenic input, fecundity was significantly enhanced. Gametogenesis and embryogenesis appear to proceed normally under potentially toxic downstream conditions. It was concluded that H. depressa may be functionally extinct in impacted areas of the Hawkesbury-Nepean River due to disappearance of fish host(s) required to complete their life cycle. This study is presented in two publications (Jupiter & Byrne 1997; Byrne 1999).

Elemental composition of tissue granules in Hyridella depressa

The aim of the microanalytical study was to identify specific tissues for monitoring metal loads in H. depressa with emphasis on the CaP granules, a major site for element uptake in freshwater mussels. These granules sequester a range of elements from the environment and in H. depressa can be sampled in mantle biopsies. Element composition in tissue granules of six populations of H. depressa were compared. For a suite of common cations including: Ca, P, Fe, Mn and Mg, granule composition reflected catchment lithology, site trophic status and indicated exogenous input. Iron was particularly important in differentiating the lake and river sites. Trace elements were also important in site differentiation and the most important were: Al, Cu, Zn and Pb. Analysis of granules in resident mussel populations may be a useful approach to assess the influence of catchment water chemistry on the bioavailability of a suite of elements to resident biota. Our results indicate that characterisation of element content of granules in resident mussel populations can provide valuable insights into animal-element interactions in freshwater systems for both ecological and ecotoxicological investigations. Use of mantle biopsy and granule analysis is a new approach to biomonitoring. Part of this study has been published (Byrne & Vesk 1996; Adams et al. 1997; Vesk & Byrne 1999).

Active biomonitoring with Hyridella depressa

An active biomonitoring study of the reproductive and bioaccumulation responses of H. depressa as tools for in-stream monitoring was undertaken in collaboration with the Centre for Ecotoxicology (NSW EPA). Mussels were placed in two streams which receive urban runoff and sewage effluent and at two control sites which had minimal urban runoff. A suite of sublethal endpoints were evaluated for the mussels following in-situ exposure, including survivorship, growth, and gonad status. H. depressa was shown to be physiologically robust exhibiting nearly 100% survival and enhanced fecundity and growth in downstream cages. Their enhanced condition appeared to reflect a response to the elevated nutrient status of receiving waters. Gametogenesis and embryogenesis proceeded normally in downstream conditions, indicating that the decline of H. depressa from the South and Eastern Creek sub-catchments is not due to reproductive failure. The tolerance of H. depressa to waters toxic to other aquatic animals, indicates that this species may be useful as in-stream monitor. The bioaccumulation response of H. depressa may be useful for detection and measurement of contaminants intractable or expensive to detect in water samples, and which most certainly have a negative impact on other organisms.

INTRODUCTION

Due to their life as sedentary suspension feeders and their impressive capacity to accumulate toxicants, bivalve molluscs play a central role in maintaining water quality in aquatic ecosystems and are often used as sentinel organisms in studies of water pollution. The use of bivalves as biomonitors is well-illustrated by the international marine 'mussel watch' program. Recent research also highlights the use of freshwater bivalves to monitor pollution in inland waters (Jeffree et al 1993; Hickey et al. 1995). Australia has a highly endemic freshwater mussel fauna and several species have been identified as important indicator organisms for monitoring levels of toxicants in impacted waterways (Jeffree et al 1993). This project investigated the reproductive and bioaccumulation responses of H. depressa as tools to monitor the health of the Hawkesbury-Nepean River system.

In parallel with the recognition of the key role that bivalves play in maintaining water quality, has come the realisation that fresh water mussels have been on the decline in the rivers of Australia for some time, apparently due to prolonged recruitment failure (Jupiter & Byrne 1997). The decline of freshwater mussels is a global phenomenon and has resulted in a significant loss in aquatic biodiversity (Bogan 1993). Conservation concerns are now focussed on identifying the causes underlying the decline and extinction of freshwater mussel species. These issues were addressed in this project through documentation of the reproductive health of populations of H. depressa from reference and impacted sites. The data was used to determine whether reproductive failure accounts for the decline of river populations and to make a predictive assessment on the long-term effects of environmental degradation on the potential for future recruitment (Jupiter & Byrne 1997; Byrne in press).

Mussels occur throughout the river systems of New South Wales and, although mussels have disappeared from impacted sites, extensive populations of H. depressa occur in impounded lakes (Byrne in press). These lake populations provided an opportunity to investigate H. depressa for active biomonitoring. Active biomonitoring involves translocation of bioindicator species from reference source populations to monitoring sites. This approach was used to investigate the reproductive and bioaccumulation responses of H. depressa as tools for biomonitoring.

Freshwater mussels accumulate metals and other elements from the environment in small CaP granules distributed through their tissues (Jeffree et al 1993). The massive contribution of CaP granules to the body mass of freshwater mussels is a unique feature of biomineralisation in these bivalves. An extensive study of the granules was undertaken to assess their potential for monitoring metal pollution. Most importantly, with respect to conservation concerns, these granules can be sampled non-destructively in mantle biopsies with no obvious deleterious effects to H. depressa (Byrne, pers obs). This study focussed on establishing methods for X-ray microanalysis (EXPMA) and secondary ion mass spectrometry (SIMS) of the granules (Byrne & Vesk 1996; Adams et al. 1997; Vesk & Byrne in press). The element composition in tissue granules of six populations of H. depressa from the Hawkesbury-Nepean River system was compared to assess the utility of this approach to assess the elemental status of the aquatic environment (Byrne & Vesk, submitted).

Specific Objectives of the Project:

1) To document the reproductive condition of Hyridella depressa from reference and impacted sites to determine how the life history stages are affected by environmental degradation.
2) To provide a predictive tool with which to assess the impact of environmental degradation on future recruitment and conservation of riverine mussel populations.
3) To quantify the reproductive output of H. depressa translocated to reference and impacted sites to assess clutch size as a bioindicator of river health.
4) To characterise the elemental profile of granules in tissue of H. depressa from reference and polluted sites in order to assess the biopsy-microanalysis method for monitoring the elemental status of the aquatic environment.

PROJECT DESCRIPTION

This project focussed on freshwater mussel Hyridella depressa and involved three areas of research: 1) reproduction of H. depressa with emphasis on the conservation biology, 2) element accumulation in tissue granules and, 3) use of the reproductive and element-accumulation responses of H. depressa for in-stream monitoring. The first two research areas were in the original proposal submitted to the Environmental Research Trust. The third aspect of the project was added in 1996 and was approved by Trust project referees.

The reproductive study was an anatomical and histological study of gonad development and embryogenesis in H. depressa. River and lake populations of H. depressa were sampled at approximately monthly intervals over two breeding seasons to document details of gametogenesis and to determine when the females incubate embryos in their gill marsupia. Four populations in Lake Burragorang and two populations in the Hawkesbury-Nepean River were studied. The study sites differed in anthropogenic influence and trophic status and the influence of these factors on reproduction in H. depressa was assessed. A skewed sex ratio in favour of females was common in the populations and histology revealed that some of these females were microhermaphrodites. It appears that sexuality in H. depressa is labile with the potential for self-fertilisation by individuals that largely function as females. H. depressa is a long-term brooder with progeny present in the marsupia for up to eight months of the year from mid winter to late summer. At the eutrophic sites influenced by anthropogenic input, fecundity was significantly enhanced and the gonads contained advanced gametes throughout the year. H. depressa at these sites had a high annual reproductive output with the females producing several clutches each year. Gametogenesis and embryogenesis appear to proceed normally under potentially toxic downstream conditions. In contrast, gametogenesis in mussels at the oligotrophic sites was more seasonal. At one of the oligotrophic sites reproductive failure was common and in 1996 most females failed to brood a single clutch. With regard to mussel populations in the Hawkesbury-Nepean River, it was concluded that H. depressa may be functionally extinct in some impacted areas due to the disappearance of the fish host(s) required to complete the life cycle (Jupiter & Byrne 1997; Byrne 1999).

The microanalytical study of element accumulation in H. depressa focussed on the CaP granules which are abundant in the tissues. Initial emphasis was placed on establishing methods for granule microanalysis. This was an extensive study comparing application of two tissue preparation techniques, chemical fixation and freeze-substitution. Although application of the routine (chemical) approach widely used in such studies would facilitate use of the granules as biomonitor, we demonstrated that this approach is prone to artefact (Vesk & Byrne 1998). Subsequently, cryo-techniques were used in a study of element levels in tissue granules from six populations of H. depressa. Our results indicate that characterisation of the content of granules in resident mussels would provide valuable insights into animal-element interactions in freshwater systems for both ecological and biomonitoring investigations.

The active biomonitoring study involved translocation of H. depressa from a source population in Lake Burragorang to monitoring sites in the Hawkesbury-Nepean River. Cages of mussels were placed in two streams, Eastern creek and South Creek which receive urban runoff and sewage effluent and at two control sites at Douglas Park and Lake Burragorang, which had minimal urban runoff. The reproductive and bioaccumulation responses of the caged H. depressa were assessed as tools for in-stream monitoring of aquatic health. H. depressa was shown to be physiologically robust exhibiting nearly 100% survival and enhanced fecundity and growth in cages deployed at downstream sites. Our results indicate that H. depressa has potential for in-stream monitoring.

PERSONNEL

The main personnel involved with this project included Dr. Maria Byrne as Principal Investigator and a full time research assistant. Mr. Moreno Julli of the Centre for Ecotoxicology (NSW EPA) was a major collaborator in the active biomonitoring program. Dr. Kathryn Prince of the Australian Nuclear Science and Technology Organisation collaborated with the SIMS study. The research assistant position was split into two half time positions because the assistants were also part-time graduate students. At the outset, these included Mr. Peter Vesk and Ms. Susan Adams. Peter worked as a half time research assistant through completion of the project in December 1997. Susan was a part time MSc student and her thesis, completed in 1996, was a methodological study of the application of SIMS and EXPMA for granule analysis. Subsequently Susan resigned and Ms. Anna Cerra worked on the project as a half time research assistant until December 1997. While Anna was on 6 months of maternity leave in 1996 assistance was provided by Ms. Maria Bucci or Ms. Paula Cisternas.

Salaried personnel to support the field component of the project was provided in-kind by Catchment Services, Sydney Water Corporation. Many volunteer divers and field assistants were also involved.

DIFFICULTIES AND DELAYS

In general the project was not hampered by difficulties or delays. A problem encountered at the outset was the inability to find recruiting populations of Velesunio ambiguus. Although our search for suitable populations of this species continued for some time, we could not locate even one. As a result, the project focussed on H. depressa. This species is abundant and also appears to be recruiting in areas of the Hawkesbury-Nepean River.

PROJECT MODIFICATIONS AND COMPARISON WITH ORIGINAL PROPOSAL

The specific objectives as listed in the original proposal (Section B1 1.2) and what was achieved are listed in Table 1. The expected outcomes as listed in the proposal (Section B1 1.3) and potential application of what was delivered are listed in Table 2. Applications of the project as listed in the proposal (B1 1.4) and comments are presented in Table 3.

EXTENSIONS TO THE ORIGINAL PROPOSED PROJECT

The active biomonitoring study was added approximately half way through the project. This study complimented the ongoing research program and provided valuable insights into the reproductive response of H. depressa translocated from oligotrophic to eutrophic conditions.

TIMELINESS

In general the research proceeded in a timely fashion and achieved all the milestones listed in section B5 5.2. Initial progress was greatly facilitated by extensive reconnaissance of the Hawkesbury-Nepean River for suitable mussel populations June to December 1994 before the project started.

Table 1. Specific objectives as listed in the proposal and what was achieved.

ORIGINAL OBJECTIVE	OUTCOME
1. To establish levels of heavy metals and other major ions in the tissues of the freshwater mussels Hyridella depressa and Velesunio ambiguus from reference and impacted sites in the Hawkesbury-Nepean River system, through the use of spectrometry and X-ray microanalysis.	Achieved for H. depressa but not for V. ambiguus for reasons stated above.
2. To assess the condition of the gonads, gametes and digestive tissue of H. depressa and V. ambiguus from selected sites through the use of histology and electron microscopy.	Achieved for H. depressa but not for V. ambiguus for reasons stated above.
3. To determine the reproductive output of H. depressa and V.ambiguus from reference and impacted sites through comparison of the number of embryos and larvae in their marsupia.	Achieved for H. depressa but not for V. ambiguus for reasons stated above.
4. To assess the long-term impact of environmental degradation on the populations of H.depressa and V. ambiguus from Hawkesbury-Nepean River with respect to potential changes in recruitment.	Achieved for H. depressa with some insights obtained for V. ambiguus.

Table 2. Expected outcomes, findings and potential application.

OUTCOME	FINDINGS AND APPLICATION
1. Establishment of the histological and reproductive responses of freshwater mussels as monitoring tools with which to assess the health of the Hawkesbury-Nepean River and other rivers in Australia.	**1.** H. depressa is highly tolerant of environmental degradation surviving in polluted waters without any obvious deleterious histological or reproductive effect. There is no obvious pathological response that can be used for monitoring. **2.** The enhanced condition of H. depressa resident and caged at eutrophic sites provided strong evidence that the bioaccumulation response of this mussel will be useful in detection of toxicants difficult to detect in routine water samples.
2. Identification of specific tissues for monitoring metal loads in freshwater mussels.	**1.** Mantle tissue has the greatest potential for monitoring tissue metal loads. This is a major site of element uptake and can be biopsied. **2.** Analysis of metal levels in mantle biopsies will be useful for monitoring metal accumulation in freshwater mussels.
3. Provision of an early detection system to assess the short-term effects of urban pollution on the mussels of the Hawkesbury-Nepean River.	**1.** Adult H. depressa did not exhibit any deleterious short-term effects in response to urban pollution. **2.** Urban pollution appears to exert its most deleterious effect on the parasitic larval and recruitment stages. **3.** The relatively rapid enhancement of body condition of caged H. depressa indicate that active biomonitoring may be a useful short-term (weeks/months) detection system.
4. Provision of a predictive tool with which to assess the long term impact of environmental degradation on future recruitment of mussels.	**1.** Assessment of gamete and embryo condition at a range of sites indicated they not effected by environmental degradation. **2.** Future recruitment of H. depressa in the Hawkesbury-Nepean Rivern will depend on the presence of host fish and suitable juvenile habitat. **3.** The impact of environmental degradation on H. depressa is clear: remaining populations in some sub-catchment areas are probably functionally extinct, adults may persist until senescence. **4.** Insights gained from this project can be used as a basis to assess and make predictions on the impact of environmental degradation on mussel populations in other NSW river systems.

Table 3. Project applications as listed in the original proposal and assessment.

APPLICATION	ASSESSMENT
1. Introduction of a new and efficient means of monitoring water quality and assessment of the long and short term environmental impacts of urban pollution in the Hawkesbury-Nepean River and other freshwater systems.	**1.** Use of H. depressa for in-stream monitoring of water quality in NSW is new. The high survivorship of this mussel in potentially toxic conditions in pilot caging study indicated that its bioaccumulation response holds promise as an efficient means to monitor water quality. **2.** For the Hawkesbury-Nepean River resident mussel populations are too disjoint and sparse for use in biomonitoring. **3.** Mussels can be translocated from sites of abundance in impounded lakes for long and short biomonitoring purposes. **4.** Decisions on the relative efficiency of mussels as biomonitors will depend on the use of field trials where the mussels are deployed in parallel with other potential biomonitors. **5.** With regard to assessment of metal levels in resident mussels, the non-lethal mantle biopsy approach may be the only acceptable means to obtain tissue for analysis from many riverine populations due to their non-recruiting status.
2. The use of freshwater mussels as indicator organisms for routine monitoring of the environmental health.	**1.** Body condition of H. depressa is sensitive to environmental trophic status. **2.** Elemental profiles of the granules reflect water metal content. **3.** This project determined and highlighted the potential application of mussel tissue responses for monitoring nutrient and metal pollution. Future research on the mussel system as a routine monitor will need to involve field trials where abiotic parameters are also measured.
3. Provision of a framework for future research programs on the status of the benthic macrobiota of the Hawkesbury-Nepean River.	**1.** Freshwater mussels are rarely if ever sampled because their habitat is not included in standard river health surveys. **2.** While benthic sampling design was not part of this project, the lack of information on freshwater mussel populations in the Hawkesbury-Nepean and other river systems is a major gap in our knowledge. Our freshwater mussel fauna is highly endemic and is an ecologically important component of our aquatic biodiversity. **3.** Assessment of the status of mussel populations in the Hawkesbury-Nepean River will depend on surveys of habitats difficult to sample such as rock crevices and submerged wood which are typically located in water of poor visibility.

OVERALL EVALUATION AND FUTURE RESEARCH

Overall, the project achieved most of the objectives outlined in the original proposal. The major exception was the inability to obtain data for Velesunio ambiguus due to the paucity of suitable populations in the Hawkesbury-Nepean River. The project provided valuable data on the reproductive, conservation and bioaccumulation biology of H. depressa. These were the main areas of emphasis and the research did not deviate from this focus. Addition of the active biomonitoring program was also important for the project. Through this program we gained considerable insights into the potential of H. depressa as an in-stream biomonitor.

Freshwater mussels are a key component of aquatic systems and by virtue of their filtration capacity can play an key role in maintenance of water quality. The major success of the project was establishing a comprehensive data base on the reproductive, conservation and bioaccumulation biology of H. depressa from reference and impacted sites the Hawkesbury-Nepean River. Prior to this project little was known about the biology of these ecologically important organisms. Considering that freshwater mussels appear to be on the decline in the river, the results of this project provide timely data on which to base decisions on their conservation and where to direct future research. Maintenance of aquatic biodiversity is the key to maintaining the integrity of river systems.

Establishment of the methods required for microanalysis of the tissue granules is a major contribution to the study of bioaccumulation in freshwater mussels and other organisms that sequester elements in concretions. This research has not been undertaken before and represents a first for Australia. Granule analysis may be particularly useful for biomonitoring when used with the mantle biopsy approach for non-destructive sampling of mussel populations. This method provides a sensible alternative to the whole-body pooled homogenate approach used by 'mussel watch'. I have no doubt that this work will attract major attention from researchers and environmental agencies overseas.

The definitive establishment of use of fresh water mussels as indicator organisms for monitoring water quality in the Hawkesbury-Nepean River could not be achieved within the framework of the research program. It would have been useful to have undertaken a parallel study of elements in mussels, water and sediment to determine how the elemental profile in the granules relates to that present in the environment. This was beyond the scope and resources of the project but would be a fruitful avenue of future research. Given the enhanced survival of H. depressa caged at sites toxic to other aquatic life, future studies of bioaccumulation in this species would be most successful if the active biomonitoring approach is adopted. Additional research in this area should involve parallel analysis of abiotic parameters.

The population ecology of H. depressa is an important area for future research. This would require reconnaissance of the Hawkesbury-Nepean River with application of SCUBA and snorkel surveys. A detailed survey is required to determine the current status of H. depressa and V. ambiguus in the Hawkesbury-Nepean River.

Finally, the filtration biology of H. depressa will also be a fruitful area for future research. Given the abundance of this mussel in key drinking water storages such as Lake Burragorang it is important to understand the contribution that resident mussel populations make to maintenance of catchment water quality.

The external referees have provided a critical assessment of the final research report submitted in June 1998.

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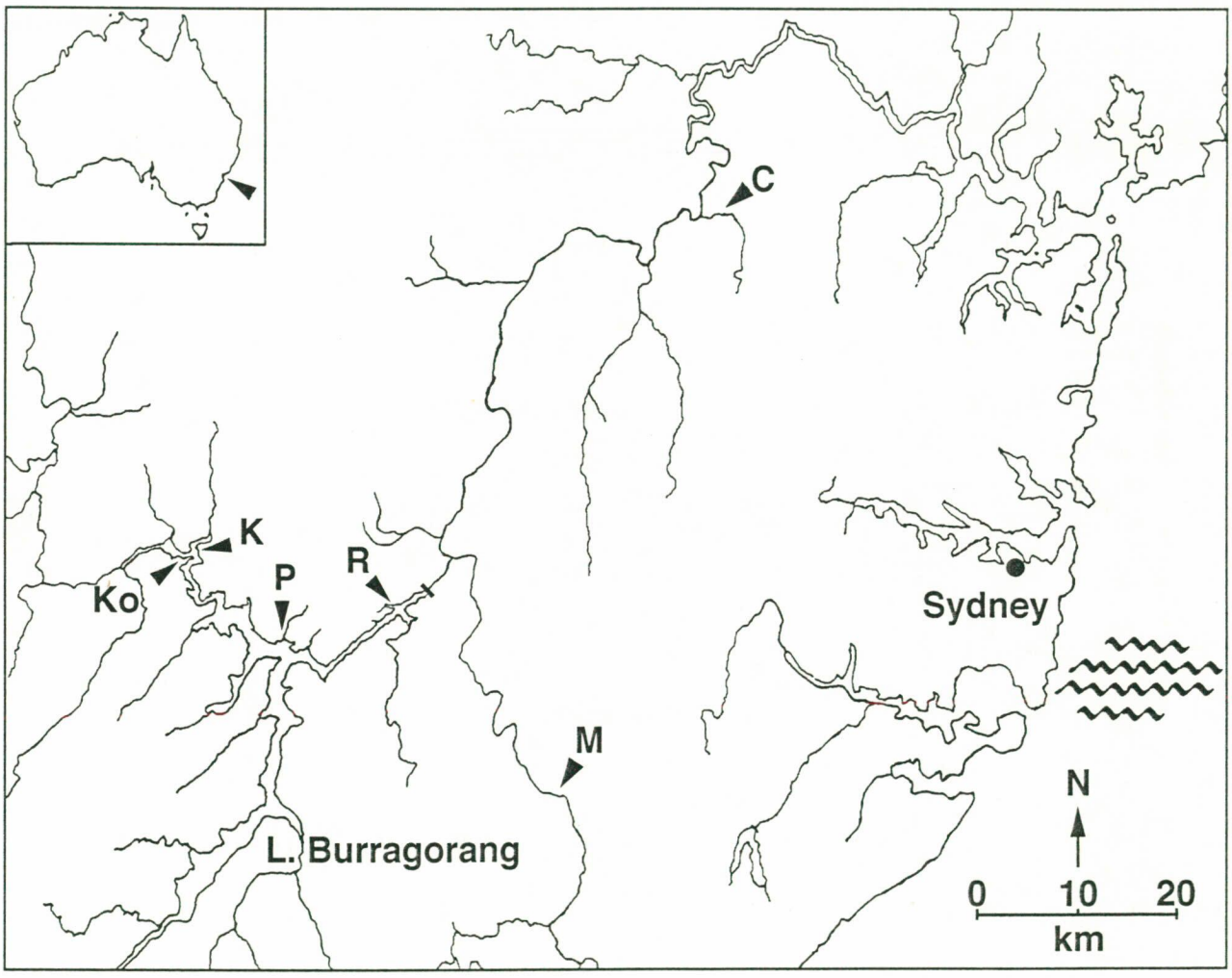
Map of the Hawkesbury-Nepean River System showing location of the study sites used for the reproductive and granule analysis study. C, Cattai Creek: K, Kedumba; Ko, Kedumba Opposite; M, Menangle Bridge; P, Pocket Creek; R, Ripple Creek. The bar adjacent to Ripple Creek denotes location of the Warragamba Dam.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Appendix 1

Invertebrate Reproduction and Development, 32:2 (1997) 177–186
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Light and scanning electron microscopy of the embryos and glochidia larvae of the Australian freshwater bivalve *Hyridella depressa* (Hyriidae)

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Summary

As is characteristic of freshwater Unionacea, the Australian species *Hyridella depressa* incubates its young within the demibranchs of its modified gills. The development of *H. depressa* was documented through light and scanning electron microscopic examination of the marsupial pouch in the inner demibranchs of brooding females. Transition from the gastrula to the glochidial stage was accompanied by a split in the larval integument. The glochidia of *H. depressa* have subtriangular shell valves each having a blunt tooth at the ventral margin. Each glochidium has a pair of hooks, one on each valve internal to the tooth. These hooks are used to attach to its fish host and are structured to interlock when the valves snap shut. One or two tufts of sensory hairs are located on the internal surface of the mantle dorsal to the hook, while a single tuft, encircled by a collar-like structure, is located centrally. The valves are pitted by pores and have concentric lines along the margins. Due to the similarity and phenotypic plasticity of adult *Hyridella* species, the morphology of their glochidia has potential for use as a taxonomic tool. In comparison to related species, the glochidia of *H. depressa* are medium-sized with a mean length of 243 μm and a mean height of 249 μm. The number of glochidia present in the gills was measured to determine the reproductive output of *H. depressa*. The embryos of an unknown mite, *Unionicola* sp., form cysts within the gill tissue of *H. depressa* and the adult mites were observed on the surface of the gills.

Key words: *Hyridella depressa*, glochidia larvae, freshwater bivalve, Unionacea

Introduction

The life history of the Unionacea is atypical of the class Bivalvia in several features (Kat, 1984). These include incubation of the embryos within modified gills and the possession of parasitic larvae modified from the free-living larvae of their marine ancestors (Kat, 1984). The complex life history of the Unionacea is well documented for several North American, African and European species in a series of studies dating back to the turn of the century (Lillie, 1895; Ortmann, 1910; Lefevre and Curtis, 1910, 1912; Coker et al., 1921; Arey, 1924; Yokley, 1972; Wood, 1974; Fryer, 1961; Kat, 1984). For the most part, these species are members of the family Unionidae. Compared with their northern hemisphere counter-

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parts, the life history of Australian hyriids is not well documented. Aspects of the reproduction and development have been described for several species (Hiscock, 1950; McMichael and Hiscock, 1958; Atkins, 1979; Jones et al., 1986). Australian unionaceans are all members of the family Hyriidae, a taxon also found in New Zealand and South America. As reported for North American and European unionids, hyriids are on the decline in Australia, with local extinctions in some river systems (Davis and Fuller, 1981; Bogan, 1993; Naiman et al., 1995). This decline highlights the need for a comprehensive understanding of all stages of their life history.

Hyridella depressa is an important component of the macrobiota of the Hawkesbury-Nepean River System of New South Wales. This species is abundant in impounded lakes, but has declined in river habitats (Byrne and Vesk, 1997). Many river populations of *H. depressa* are comprised only of large adults, indicating that recruitment has not occurred for some time (M. Byrne, pers obs). In this study, the structure of the developmental stages of *H. depressa* was documented by microscopic examination of the brood pouch in the gills. Particular attention was paid to structures associated with the glochidial parasitic stage, including the attachment hooks and sensory hairs. Due to the phenotypic plasticity of unionaceans and the similarity between congeneric species (Rand and Wiles, 1982; Jones et al., 1986; Pekkarinen and Englund, 1995a), these larval structures have potential for use as taxonomic characters to discern between *Hyridella* species. The structure of the marsupial gill was also examined and the reproductive output of *H. depressa* was determined by counting the number of glochidia in the brood pouch.

Materials and Methods

H. depressa was collected from four sites on Lake Burragorang in New South Wales from October 1995 to March 1996. Two of these sites were at the Cox River arm of the lake near the Kedumba River (33°51′S; 150°20′E), one at Pocket Creek (33°55′S; 150°25′E), and one at Ripple Creek (33°53′S; 150°35′E). Specimens were retrieved from depths of 3–5 m by SCUBA. The shell dimensions of adult females were measured with vernier calipers.

For light microscopy, the gills were excised from mussels and fixed in 10% formalin. Portions of the gills were dissected, dehydrated in a graded series of ethanols and embedded in paraffin. Serial sections (5 μm thick) were stained with hematoxylin and eosin.

For scanning electron microscopy, dissected gills were fixed in 2% glutaraldehyde in 0.1 M Sorenson's sodium phosphate buffer (pH 7.2) at room temperature for 1 h. After rinsing with several changes of buffer, the gills were cut in cross section to facilitate visualization of the glochidia within the brooding channels. Glochidia were removed from some of these gills with forceps and transferred to mesh baskets for post-fixation. Specimens were post-fixed in 2% cacodylate buffered (pH 7.35) osmium tetroxide at 4°C for 1 h and washed with several rinses of 0.2 M cacodylate buffer. The specimens were dehydrated in graded ethanols and critical point dried. Some gill tissue that had been preserved in 10% formalin was rinsed several times in 0.2 M Sorenson's buffer and then post-fixed and dried as above. Mites that had been placed in mite fix (a 5:4:1 solution of glycerine, distilled water and glacial acetic acid, respectively) were also post-fixed and dried as above. The specimens were mounted on aluminum stubs, sputter-coated with gold-palladium, and examined with a Phillips 505 scanning electron microscope. Glochidial shell measurements were taken from micrographs with care taken to ensure nearly identical orientation. The length was measured as the maximum valve diameter in the hinge plane and height was measured as the depth from hinge to beak (Jones et al., 1986).

For fecundity measurements, the gills of 18 brooding females were washed three times in distilled water to remove traces of formalin, and the glochidia were removed from the brooding pouch by teasing and agitation with forceps. Once removed from the brooding canals, the glochidia were pipetted into a beaker. Distilled water was added to make a total volume of 100 ml. The glochidia were suspended in the solution with a magnetic flee rotating at 2,000× per minute. For each sample, the number of glochidia was counted in ten replicates of 100 μl aliquots.

Results

Structure of the gills and embryos

The embryos of *H. depressa* develop in a marsupial pouch located in the middle third portion of the inner demibranchs of the gills of females (Figs. 1a–e). In this portion of the gills, the interlamellar septa are thickened and more prominent compared with the non-marsupial region of the gill. These septa provide structural support for the developing young (Figs. 1b, d,e, 2a). They are a permanent feature and, in non-brooding specimens, allow for rapid identification of females.

The embryos filled the primary water canals of the pouch, the walls of which bulged outwards due to the large volume occupied by the young. The canals are

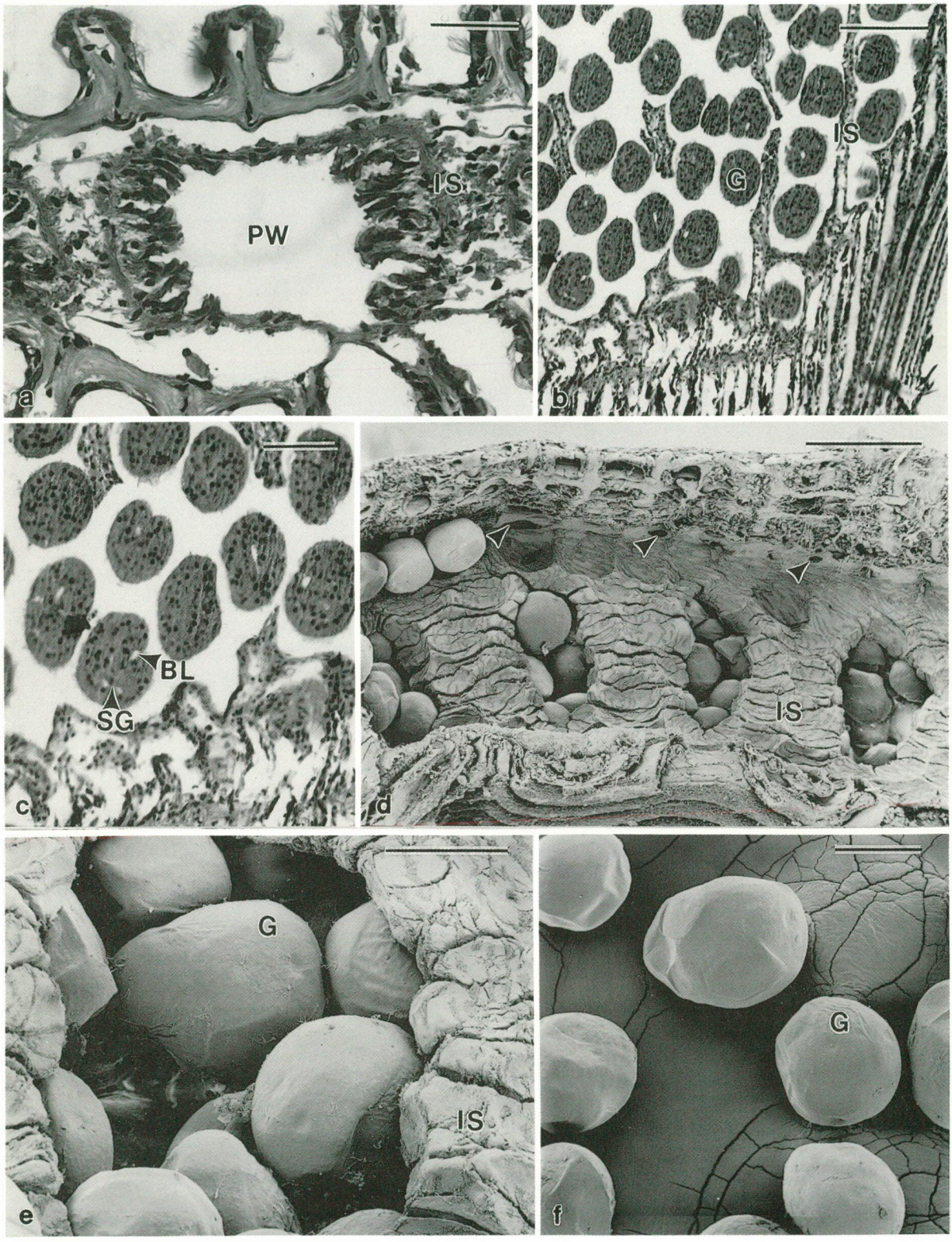
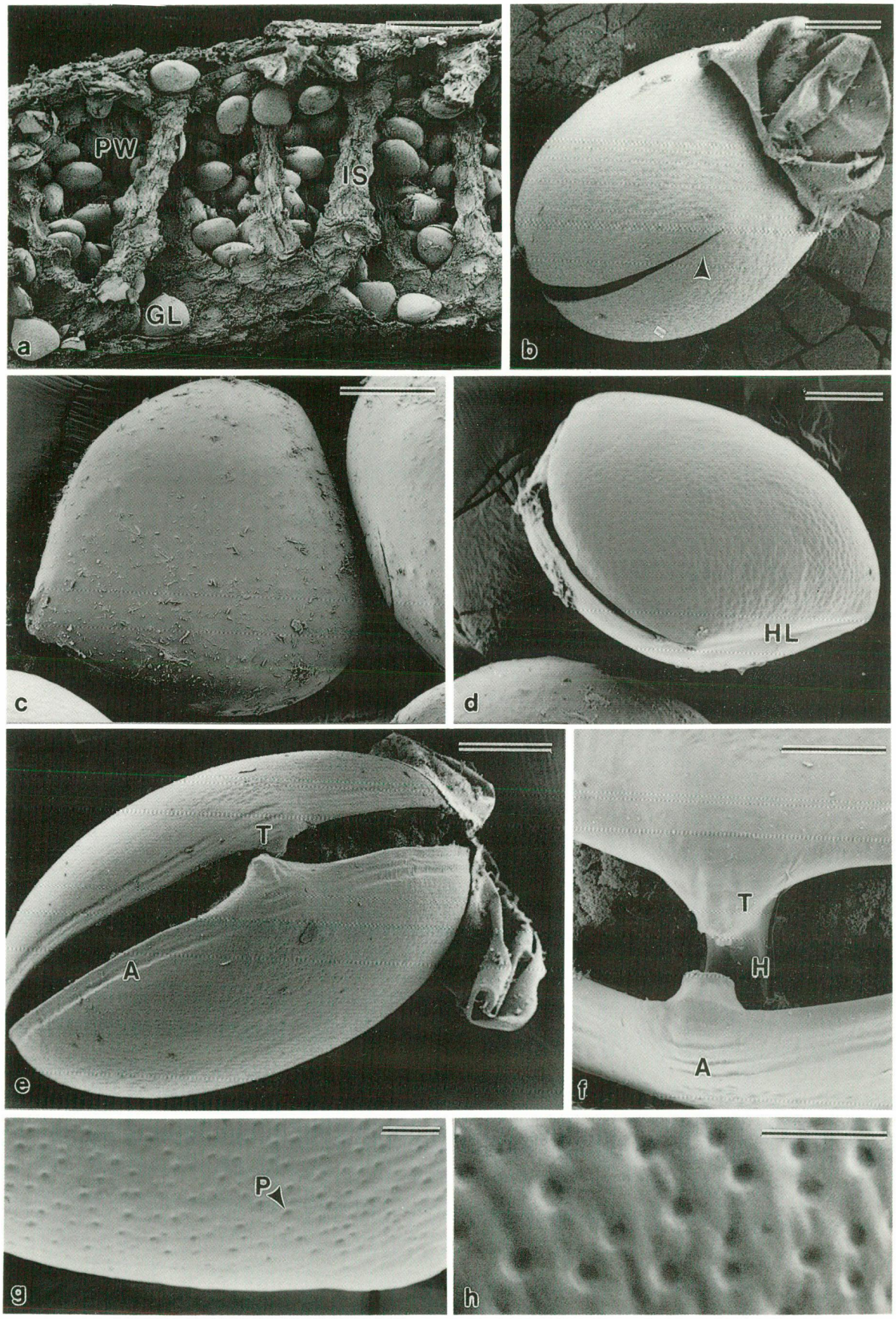


Fig. 1. Light (a–c) and scanning electron microscopy (d–f) of the marsupial pouch of *Hyridella depressa*. a: Primary water canal (PW) within the inner demibranchs of a non-brooding female. b–e: Brooding female with gastrulae (G) in the marsupium. Arrowheads, possible secondary water channel; BL, blastopore; IS, interlamellar septa; SG, shell gland. f: Gastrulae (G) with wrinkled vitelline membranes. Scale bars: a: 45 μm; b,d: 300 μm; c: 150 μm; e,f : 100 μm.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separated by interlamellar septa (Fig. 1d). Small compartments lateral to the water canal may be potential sites for secondary water canals (Fig. 1d). The gastrulae are round to oval in shape (Fig. 1b,c) and have a mean diameter of 151 μm (SE=3.16 μm, n=10). In sections, these embryos consist of a solid ball of cells except for two invaginations which form the blastopore and shell gland (Fig. 1c). The vitelline membranes of the embryos become wrinkled during the drying process (Fig. 1f). Gills incubating early embryos are readily identified by the white to cream color of the brood mass. The embryos increase in size as they developed to the glochidia stage and the interlamellar septa become thinner as the volume occupied by the brood mass increases (Fig. 2a).

Structure of the glochidia

Initially the glochidial shell is thin and transparent. As the larvae develop, the shell becomes thicker, translucent and light brown in color. This results in the darkening of the color of the marsupial pouch. As the glochidium develops, a ventral split occurs in the integument along the line of the ventral mantle edge (Fig. 2b). This change is evident when the early embryos are compared with a fully developed glochidium (Fig. 2b,d).

The glochidial valves are subtriangular in outline with a straight hinge line and a blunt tooth at the ventral margin (Figs. 2c–f). In closed glochidia, these teeth meet in the midline or slide past each other to interlock (Fig. 2e,f). The larvae had a mean length of 243 μm (SE=5.38 μm, n=5) and a mean height of 249 μm (SE=1.79 μm, n=5). Along the ventral valve margin, 2–3 concentric lines are present (Fig. 2e,f). The valve surface is pitted with pores (Fig. 2g,h).

Once fully developed, the glochidia hatch from their membranes and emerge with their valves open, exposing the hooks, the larval mantle, sensory hairs and the larval adductor muscle which extends between the two valves (Fig. 3a). On each valve a hook is located dorsal to the tooth (Fig. 3b,c). One hook is slightly recurved towards the adductor muscle (Fig. 3b), while the opposing hook has a distinctive groove (Fig. 3c), thus allowing the pair to interlock when the valves snap shut. The hooks measure 44.4 μm (SE=0.002, n=2) from base to tip. They lack surface spines. On the inner mantle epithelium of each valve, a single tuft of sensory hairs is centrally positioned in the dorsal region (Fig. 3e). A collar-like structure encircles the hairs at the base of the tuft (Fig. 3e). One or two tufts of sensory hairs are also found near the ventral margin of each valve internal to each hook (Fig. 3d). There were no collar-like structures associated with these tufts.

In aquaria, the glochidia of *H. depressa* are discharged as individuals. No larval threads or conglutinates were observed. Discharged glochidia typically have their valves open and exhibit periodic spontaneous snapping behavior, rapidly shutting and opening their valves. This response is readily elicited by tactile stimuli from a probe.

Fecundity

The total number of glochidia found in the inner demibranchs varied considerably, ranging from 1,900 to 62,800. The specimens grouped into two categories: those from Pocket Creek and Ripple Creek were smaller (42–50 mm shell length) and contained fewer glochidia ($\bar{x}$=7088; SE=1455; n=8) compared with the specimens from Kedumba which were larger (53–61 mm shell length) and had a greater mean brooding capacity ($\bar{x}$=41,880; SE=4,243; n=10). In general, there was a positive correlation between the number of glochidia and the drained weight of the adult female (r^2=0.814) (Fig. 4a) and between the number of glochidia and the adult shell length (r^2=0.596) (Fig. 4b).

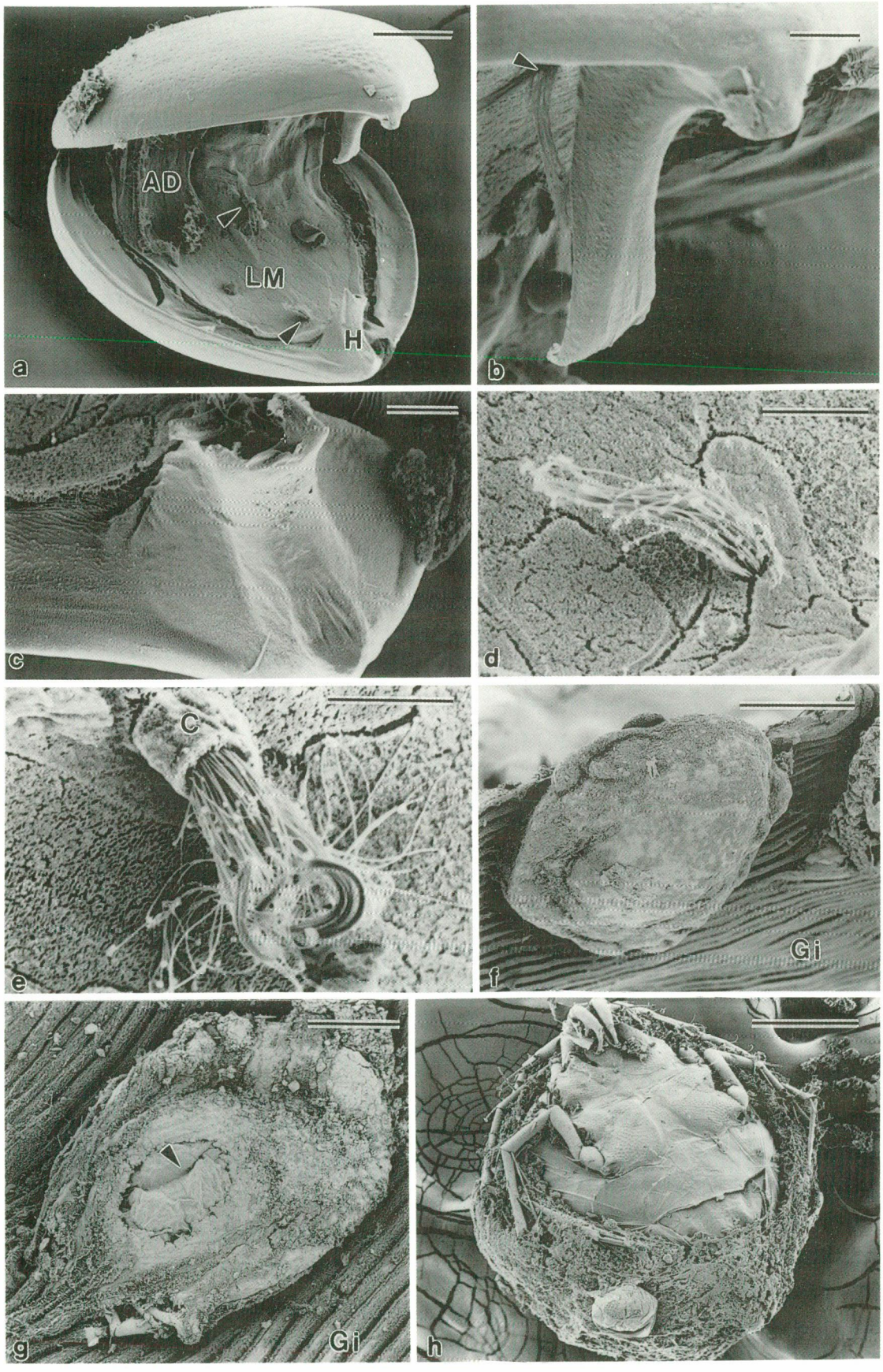
Associated mites

Several specimens of *H. depressa* were infested by an unknown species of mite, *Unionicola* sp. (Figs. 3f–h). The female mites deposit their eggs on the gills and the mite embryos develop embedded within the gill tissue (Fig. 3f,g). Gill tissue appears to grow around these embryos to encapsulate them (Fig. 3f,g). Juvenile and adult mites were often seen on the gill surface. It is not known if the mites have an effect on the reproduction of the host.

Discussion

Unionaceans are categorized according to the organization and position of the brood pouches. Incubation in all four demibranchs with lamellae

Fig. 2. Scanning electron microscopy of the glochidia of *Hyridella depressa*. a: Glochidia (GL) in the primary water canals (PW), which are separated by interlamellar septa (IS). b: Embryo in transitional stage with the mantle lobes being formed by invagination of the ventral plane (arrowhead). c,d: The valves are subtriangular and have a straight hinge line (HL). e,f: The valve margins have blunt teeth (T) and annular lines (A). H, hooks. g: Pitted surface of the shell. P, pore. h: Details of pores. Scale bars: a: 300 μm; b,d,e: 50 μm; c: 70 μm; f: 20 μm; g,h: 10 μm.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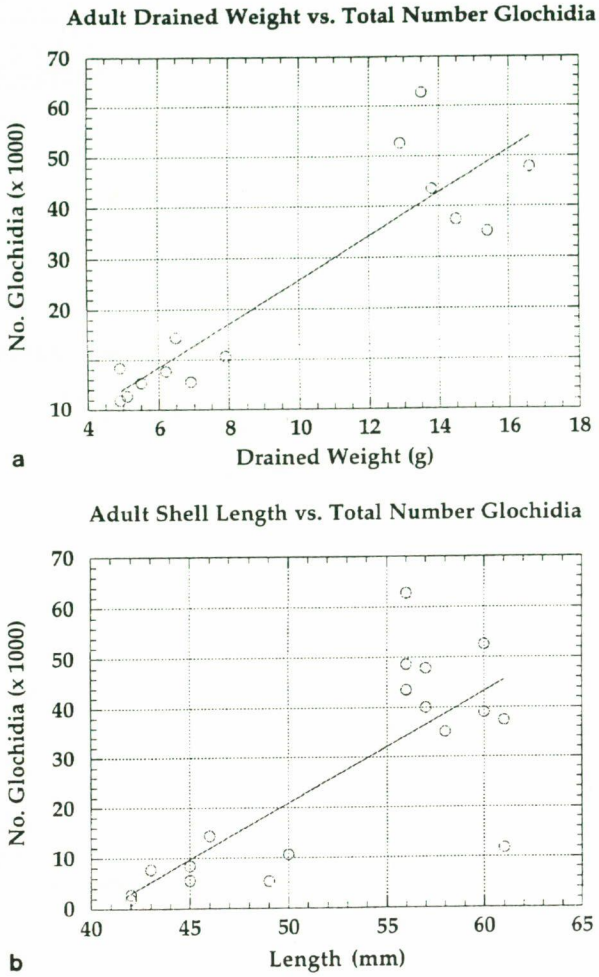

Fig. 4. a: Relationship between the number of glochidia in the inner demibranchs of *Hyridella depressa* and adult drained weight. The cluster of data points on the left are from the small mussels from Pocket Creek and Ripple Creek and the points on the right are from the Kedumba mussels. $y = 4.303x - 17.374$; $r^2 = 0.814$; $p = 0.0001$. b: Relationship between the number of glochidia and adult shell length. $y = 2.236x - 90.84$; $r^2 = 0.596$; $p = 0.0002$.

Fig. 3. (opposite): Scanning electron microscopy of hatched glochidia of *Hyridella depressa* and mites, *Unionicola* sp., associated with the gills. a: Hatched glochidium with open valves displaying attachment hooks (H), sensory hair tufts (arrowheads), adductor muscle (AD) and larval mantle (LM). b: Recurved hook and sensory hair tuft (arrowhead) behind the beak. c: Overhead view of opposing hook with groove to accommodate complementary recurved hook. d: Sensory tuft near valve margin behind hook. e: Sensory hair tuft with a collar-like structure (C) at base near hinge. f: Cyst containing embryo of *Unionicola* sp attached to the gill (Gi) tissue of *H. depressa* and surrounded by host tissue. g: Mite cyst with an opening showing embryo inside (arrowhead). Gi, gill. h: Underside of mite embryo removed from gill showing appendages. Scale bars: a: 50 μm; b–e: 10 μm; f: 300 μm; g,h: 200 μm.

separated by connective tissue is considered to be the most primitive condition (Davis and Fuller, 1981; Kat, 1984; Richard et al., 1991). Evolutionary modifications to the primitive brooding plan involved acquisition of marsupia restricted to the inner or outer demibranchs only or to distinctive positions within the demibranchs (Davis and Fuller, 1981; Richard et al., 1991). In *H. depressa* the marsupia are restricted to the midportion of the inner demibranchs and the interlamellar septa are distinctly thickened. The structure of the gills of *H. depressa* reflects the advanced condition. Additional information needed to categorize the gill specializations exhibited by *H. depressa* include: the number of interlamellar septa, the magnitude of swelling during development, and the presence and arrangement of secondary water channels (Kat, 1984; Tankersley and Dimock, 1992). The small spaces alongside the water canals in brooding *H. depressa* may be secondary water channels, but it is not known if these are homologous to those in the Unionidae. In unionid species these channels serve to maintain water transport within the gill during the brooding season (Tankersley and Dimock, 1992).

The number of glochidia produced by unionaceans decreases as the marsupia become more specialized (Kat, 1984). *Leptodea fragilis*, which incubates in the entire outer demibranchs, contains approximately 2,225,000 glochidia, while *Lampsilis siliquoidea*, which restricts incubation to the posterior portion of the outer demibranchs, contains approximately 129,000 glochidia (Kat, 1984). The lower numbers of glochidia found in the gills of *H. depressa* reflects the restricted allocation of gill space to the brood pouch. In addition, *H. depressa* is considerably smaller than the unionids. The capacity of the brood pouch in *H. depressa* is also related to the size of the adult. The strong positive correlation between the number of glochidia and the adult body weight was expected because the glochidial mass was part of the weight measure. A more independent and positive correlate is provided by the adult shell length.

There have been numerous speculations on the adaptive advantages of brooding versus broadcasting in unionaceans. For small bivalves, Sellmer (1967) proposed that brooding is a life history strategy to increase the survivorship of a few offspring. This explanation however, cannot be applied to unionaceans, which are large and highly fecund. Unionaceans present a major exception to the general association between small adult size and brooding in the Mollusca (Kat, 1984). The rationale underlying this exception is not yet understood although is considered in several studies (Kat, 1984). Potential advantages for brooding include protection from unfavorable

environmental conditions and predators (Wood, 1974; Richard et al., 1991; Tankersley and Dimock, 1992). It has been suggested that the change from marine to freshwater was associated with a switch to brooding to prevent loss of the larvae by being swept downstream (Davis and Fuller, 1981). Freshwater habitats are more unpredictable than marine habitats and experience greater fluctuations in current flow and temperature. In Australia, freshwater bivalves appear well-adapted to extremes of drought and flood (Walker, 1981). It is also suggested that brooding is beneficial because larval release is triggered by the onset of favorable environmental conditions (Richard et al., 1991). Atkins (1979) found that peaks of glochidial parasitism of fish by *H. drapeta* corresponded to an annual rise in water temperature and suggested that temperature change may provide a mechanism for the timed release of larvae. Brooding is also considered to be advantageous for dispersal by habitat-specific fishes, thereby reducing the unpredictability of freshwater habitats (Kat, 1984).

Isolation of the embryos within the brooding chamber of freshwater mussels is also suggested to be beneficial because it facilitates the transfer of maternal nutrients to the developing larvae (Silverman et al., 1985,1987; Richard et al., 1991; Tankersley and Dimock, 1992). This adaptation is considered to be particularly important with respect to the low ion concentrations characteristic of freshwaters (Silverman et al., 1985,1987; Richard et al. 1991; Tankersley and Dimock, 1992). Structural modifications in the brooding gills support the suggestion of maternal transfer. As the embryos fill the demibranchs, they become physically separated from the water in the mantle cavity (Richard et al., 1991). Although the developing young are isolated from nutrients cycling through the mantle cavity, their intimate association with the interlamellar septa provides a surface for transfer of maternal nutrients to the embryos (Richard et al., 1991; Tankersley and Dimock, 1992). The pores of the glochidial shell may also serve as a site for nutrition and gas exchange (Rand and Wiles, 1982; Pekkarinen and Englund, 1995b). Evidence for extraembryonic investment by the adult was provided by Silverman et al. (1987) who documented incorporation of maternal calcium into the larval shells of *Anodonta* and *Liguma* species. Autoradiographic evidence for transfer of maternal nutrients from the parent to the developing embryos was also provided by Wood (1974). Although there appears to be good evidence for the maternal support hypothesis, it is likely that the switch to brooding by freshwater mussels was also associated with the need for protection from environmental extremes.

The morphological differentiation that accompanies the development to the glochidial stage is strikingly different to that seen for the larvae of marine bivalves. Lillie (1895) was among the earliest to note that the transition from the solid blastula to a bivalved glochidium results from an invagination of the ventral surface along the median plane marking the differentiation of the mantle lobes. This change was also observed in the larvae of *H. depressa*.

Prior to release, the glochidia hatch from the vitelline membrane and pass from the marsupium through the suprabranchial canal. They are extruded through the excurrent siphon (Kat, 1984). To increase the probability of successful infection of a fish host, many freshwater mussels package their larvae in a remarkable array of fish-attracting mucus conglutinates that vary in shape and color between species (Kat, 1984). Unlike *H. australis*, which extrudes a tan, wormlike conglutinate (Jones et al., 1986), *H. depressa* was not observed to release a conglutinate. Many species of glochidia also have a larval thread which is thought to bind them together and assist in attachment to the host (Wood, 1974; Atkins, 1979; Rand and Wiles, 1982). Like other *Hyridella* species, *H. depressa* lacks a larval thread; this is an important taxonomic character for the genus (H.A. Jones, pers comm).

Like the glochidia of *H. depressa*, other unionacean larvae have hooks and sensory hairs which are specialized to sense and aid in attachment to the fish host. The hooks function in attachment, facilitate encystment by irritating the host tissue and may serve to loosen the tissue for nutrition (Kat, 1984; Pekkarinen and Englund, 1995a). The structure of the hooks differs between species and these differences appear to reflect the range of host tissue, including the gills, fins, and scales, to which they attach. For example, Pekkarinen and Englund (1995b) report that the large glochidia of *Anodonta*, with their powerful hooks, were successful in attaching to tougher fish tissue, such as fins, whereas the small glochidia of *Unio*, with their more delicate hooks, fared better by penetrating softer tissue, such as gills. It is suggested that these morphological differences enable co-occurring species to occupy different niches and parasitize different fish species within the same habitat (Pekkarinen and Englund, 1995b). The interlocking hooks of the glochidia of *H. depressa* are probably specialized for attachment to the external tougher tissues of their fish hosts. The glochidia of *Alathyria jacksoni*, a hyriid species with similarly sized and shaped hooks as the glochidia of *H. depressa*, usually parasitize the mouth and fins of their fish hosts (Walker, 1981).

Although the glochidia of *H. depressa* and other species exhibit attachment behavior in response to general tactile stimuli, chemical stimuli from a fish host is required for encystment (Wood, 1974; Kat, 1984). Host substances, such as fish blood, are recognized by the tufts of chemosensory hairs (Pekkarinen and Englund, 1995b). These hairs are thought to stimulate prolonged contraction of the adductor muscle until the glochidia becomes encysted (Wood, 1974). The number and arrangement of the sensory hair tufts are variable between species. In *Anodonta*, *Pseudoanodonta* and *Unio* species, each glochidial valve has one dorsal tuft near the hinge and three ventral tufts in assorted arrangements immediately internal to the tooth (Pekkarinen and Englund, 1995b). *H. depressa* also has a dorsal sensory tuft and one or two ventral tufts per mantle lobe. The presence of a collar-like structure distinguishes the dorsal tuft from the tufts at the ventral shell margin. The exact number of ventral tufts in *H. depressa*, however, is not known due to the difficulty of fixing glochidia with their valves open. Further examination may reveal that the number of ventral tufts is also variable in this species.

Jones et al. (1986) describe the glochidial hooks of Australian hyriids as distinct from those seen in North American unionaceans and contend that the family Hyriidae may require further subdivision on the basis of hook structure alone. Unlike the hooks of *Anodonta* and *Unio* species, the hooks of *H. depressa* do not possess spines (Pekkarinen and Englund, 1995b). Rather, they are modified morphologically to fit together in an interlocking manner, enabling the glochidia to ensnare a piece of fish tissue. Glochidial size may also be useful as a taxonomic character to distinguish between *Hyridella* species (Table 1) (Jones et al., 1986). The glochidial dimensions reported here for *H. depressa* are similar to those reported by Jones et al. (1986). As found in unionid species (Pekkarinen and Englund, 1995a), the suite of glochidial characters that have potential for taxonomic identification of *Hyridella* species include size, tooth structure, hook structure and the number and location of the sensory hair tufts.

Table 1. Comparison of the height and length of the glochidia of *Hyridella* species

Hyridella species	Glochidial height (μm)	Glochidial length (μm)	Source of data
H. australis	94.7	73.9	Jones et al. (1986)
H. depressa	244	253	Jones et al. (1986)
H. depressa	249	243	This study
H. drapeta	230	330	Atkins (1979)

Although unionicolid mites are commonly encountered in freshwater bivalves, it is not known how they impact on reproduction (Dimock, pers comm). The number of mites associated with a single specimen varies considerably and species of mites are known to be tissue specific (Dimock, pers comm). In *H. depressa,* the mites utilize gill tissue for oviposition. In some specimens a large proportion of the gill surface was occupied by mite embryos (Byrne, pers obs) and, in this situation, the mites probably exert a deleterious effect on filtration food supply and reproduction of the host (Dimock, 1988).

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MICROANALYSIS OF ELEMENTS IN GRANULES IN *HYRIDELLA DEPRESSA* (BIVALVIA): MULTIVARIATE ANALYSIS AND BIOMONITORING POTENTIAL

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ABSTRACT

The Australian freshwater mussel *Hyridella depressa* accumulates elements in extracellular granules distributed throughout its tissues. This species occurs in the Hawkesbury-Nepean River System and its potential as a tool to assess the elemental status of the aquatic environment was examined in an X-ray microanalytical investigation of the granules. Granule microanalysis is a new approach to biomonitoring and emphasis was placed on the analytical and statistical methods required. Element levels in granules in freeze substituted tissue were documented for three animals from four sites in Lake Burragorang. Microanalysis was performed in a scanning transmission electron microscope equipped with an energy dispersive X-ray detector. Analysis of the data by Non-metric Multi-Dimensional Scaling and ANOSIM indicated that the sites differed and that animals within sites also differed. This research indicated that X-ray microanalysis of elements in the granules has potential for biomonitoring, particularly in conjunction with multivariate analysis. It also indicated the requirement to increase the number of animals examined from each site and highlighted the need to use several reference sites in biomonitoring studies.

Key words: environmental monitoring, freshwater mussel, MDS, X-ray microanalysis

INTRODUCTION

Bivalve molluscs are a recognised tool for monitoring water quality and pollution in aquatic systems because they accumulate and detoxify heavy metals in their tissues (Phillips and Rainbow, 1993; Jupiter and Byrne, 1997). In the river systems of Australia, mussels of the family Hyriidae are an important component of the macrobiota (McMichael and Hiscock, 1958). These mussels sequester elements from the environment in small calcium phosphate granules distributed throughout the tissues (Jeffree *et al.*, 1993; Adams *et al*; 1997). The mussel *Hyridella depressa* occurs throughout the Hawkesbury-Nepean River System of New South Wales and is the dominant macroinvertebrate in Lake Burragorang. This lake was created by construction of the Warragamba Dam and is the major source of drinking water for Sydney.

In this study, the element content of the granules in freeze-substituted tissue of *H. depressa* from four sites in Lake Burragorang was examined with energy dispersive X-ray microanalysis. Preliminary analysis revealed that the concentrations of elements in the granules varied in a way which was neither obvious nor consistent. No single element was useful in distinguishing samples from different sites. Consequently, a multivariate approach was used to analyse the data. The main objective of the research was to assess the accumulation of elements by the tissue granules of *H. depressa* as a tool with which to monitor the health of the Hawkesbury-Nepean River and to determine the optimal microanalytical and statistical methods.

MATERIALS AND METHODS

Study sites

Lake Burragorang, is a relatively clean environment and was chosen as a source of reference sites for collection of *H. depressa*. Four sites were sampled: Ripple and Pocket Creeks and two sites at the confluence of the Kedumba River and Coxs River arm of the lake. The first two sites are pristine and the latter two are eutrophic. The Coxs River drains agricultural lands and small rural communities and the Kedumba River receives effluent from the South Katoomba sewage

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treatment plant. At each site the largest specimens were collected for analysis. The shell lengths of mussels collected from Ripple Creek, Pocket Creek, Kedumba upstream and Kedumba opposite were: 45-50 mm, 45-50 mm, 55-60 mm and 55-60 mm, respectively.

Specimen preparation

H. depressa were collected by divers and transported to the laboratory in plastic bags filled with lake water. To avoid sources of variation such as season and sex, the study was restricted to males collected during the non-breeding period in April-May 1995. Mussels were prised open, and males were selected by microscopic observation of the gonads. Mantle tissue from three specimens from each site was cryofixed with copper-tipped pliers, cooled to liquid nitrogen temperature (-196°C). The tissues were freeze-substituted (Edelmann, 1990) in acetone at -80°C for 3 days, -20°C for 16 h, 4°C for 4 h, rinsed with fresh acetone twice, and infiltrated with Spurr's resin over 4 h to 30% resin in acetone and left overnight. Resin concentration was increased to 70% for 24 h, then 100% resin for 24 h before final embedding in fresh resin and polymerisation at 60°C for 24 h. Sections (0.5 µm thick) were cut dry for microanalysis and transferred to coated nickel slot grids. Energy dispersive X-ray microanalysis was performed in a Philips CM12 scanning transmission electron microscope operated at 120kV equipped with an Edax X-ray detector.

X-ray Microanalysis

The granules were analysed using a small area raster, approximately 360 x 360 nm. For each specimen, five granules were analysed from each of 4 granule aggregations. Analyses lasted 100 live seconds. Data on 11 elements, Ca, P, Fe, Mg, Mn, Al, Cu, Ba, Zn, S and Pb were collected. For each element the number of X-ray counts per second in peaks was calculated after background stripping.

Data Analysis

Samples were ordinated with non-metric multi-dimensional scaling (MDS) (Kruskal and Wish, 1978; Clarke, 1993) using PRIMER software (Plymouth Marine Laboratory, UK). This technique was chosen because of its lack of assumptions about the data, such as multivariate normality and homogeneity of variances and covariances, relying only on a matrix of rank similarities (a is more similar to b than a is to c, etc) and because it preserves the relationships between samples in low dimensions. By contrast, other ordination techniques such as principal components analysis and factor analysis often require several dimensions to represent the relationships between the data (Clarke, 1993).

Data for ordination were standardised by summing the counts in an analysis and dividing the counts for each variable (element) by that total. This removes the effect of total count variation from analysis to analysis which may occur due to variation in section thickness and beam current. Data were transformed to square roots to prevent the ordination representing only the most common elements. The effect of other transformations including logarithmic, and fourth root were also examined. Bray-Curtis similarities were used (Clarke, 1993). The stress value is a badness-of-fit measure. It indicates how well the distances among samples as plotted represent the calculated distances in the dissimilarity matrix. As a rule of thumb, stress values of 0.2 are reliable though details should be interpreted with caution. Stress values less than 0.1 are highly reliable (Clarke, 1993). Nested analysis of similarities (ANOSIM) was performed using granule analyses from mussels at different sites. These similarities are compared within and between groups defined *a priori* in a constrained randomisation test (Clarke and Green, 1990; Clarke, 1993). Determination of elements responsible for the dissimilarities between groups was performed with the program SIMPER in the PRIMER package, allowing univariate methods to be applied to selected element data.

For analysis of individual elements, plots of log standard deviation against log mean indicated that square root transformation of the data would stabilise variance heterogeneity and induce normality (Clarke and Green, 1988). Mean X-ray counts of individual elements were calculated for each site and plotted with bars representing SEM (n = 3 animals per site).

RESULTS

Scanning transmission electron micrographs (STEM) of unstained 0.5 µm thick sections of the mantle of *H. depressa* show that the tissue contains large aggregations of electron-dense extracellular granules (Figure 1). These granules are spherical and range in diameter from 0.5 µm to 1.0 µm. A typical X-ray spectrum from an analysis of a granule is shown in Figure 2. The highest peaks are generated by Ca, P, and Fe. Minor peaks for other elements including Mn and Ba are also visible. The Ni peak is from the specimen support grid.

Ordination of data on 11 elements from the three animals from each site by non-metric MDS indicates the separation of the two Kedumba sites from the Ripple and Pocket sites (Figure 3). ANOSIM determined that sites differed ($p < 0.02$) but the sample size was not large enough for pairwise comparisons at the $p = 0.05$ level of significance. Mussels within sites also differed ($p < 0.001$). A sample of 5 *H. depressa* per

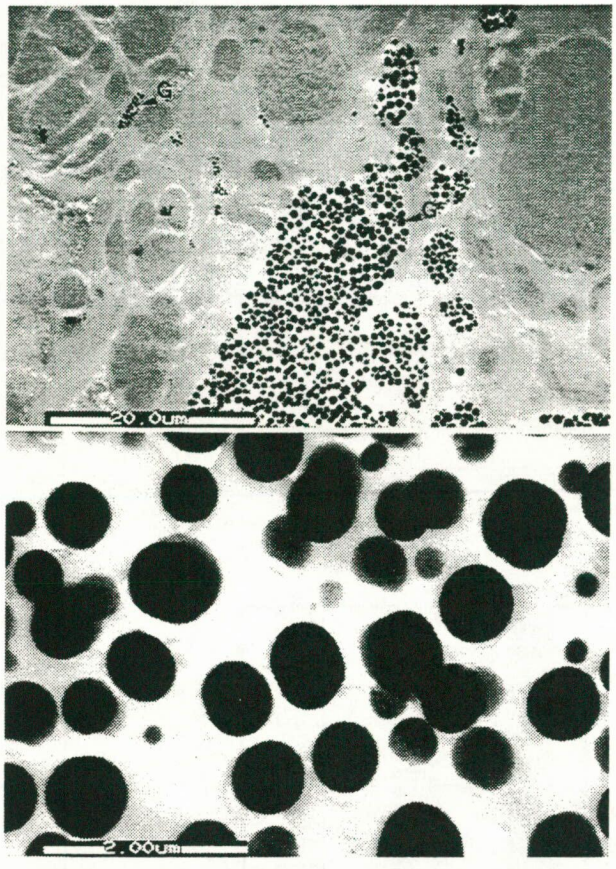

Figure 1. *STEM micrographs showing aggregations of extracellular calcium phosphate granules (G) in the mantle of Hyridella depressa.*

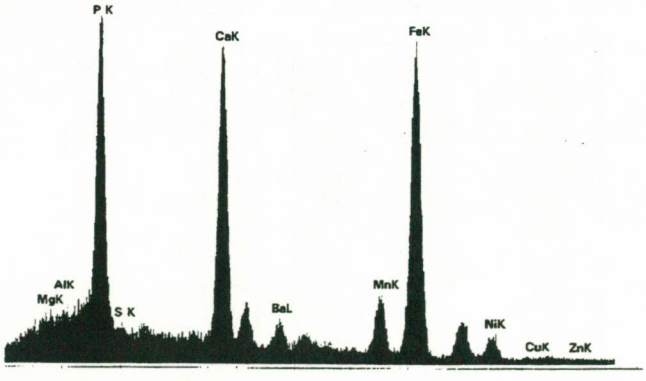

Figure 2. *Typical X-ray spectrum generated by analysis of a granule in the mantle of Hyridella depressa. Note the distinct Ca, P and Fe peaks. K and L refer to X-ray lines.*

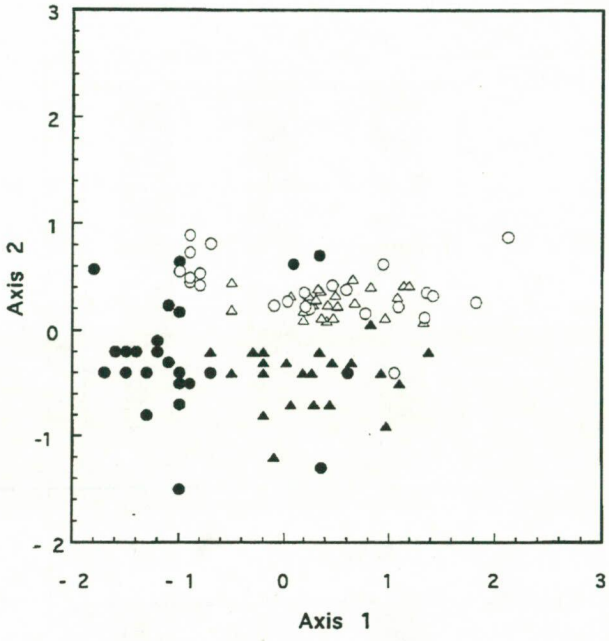

Figure 3. *MDS ordination of square root transformed standardised X-ray counts from the granules in three Hyridella depressa from four sites in Lake Burragorang. Bray-Curtis similarities, Stress = 0.13. Filled triangles, Kedumba Opposite; Filled circles, Kedumba Upstream; Open triangles, Pocket Creek; Open circles, Ripple Creek.*

site (12 granules per specimen) is expected to be sufficient, but pairwise comparisons using a Bonferroni correction for experimental type I error rate would be facilitated by a sample size of 6.

Analysis of the data with SIMPER revealed that the important elements for determining the similarity or differences among groups varied according to what transformation of the data was used (Figure 4). For untransformed data, dissimilarity among sites was defined chiefly by the most common elements, Fe, Ca and P. This was also the case for square root transformed data, with Mg and Mn also becoming important. Square root transformation allowed representation of all 11 elements in the analyses without putting disproportionate weight on the most abundant (Ca, P, Fe) or the rarest (Al, Cu, Zn, Pb) elements. Logarithmic transformation increased the importance of the rarer elements. SIMPER analysis of log transformed data showed that the dissimilarity between the Kedumba sites and the Pocket and Ripple Creek sites was due to Mg, Al, Mn, P, S and Ba, in that order (Figure 4). Deciding on the appropriate transformation depends on the elements of interest, on examination of the whole data set and on understanding how the transformation affects the data (Figure 4). For instance, trophic status

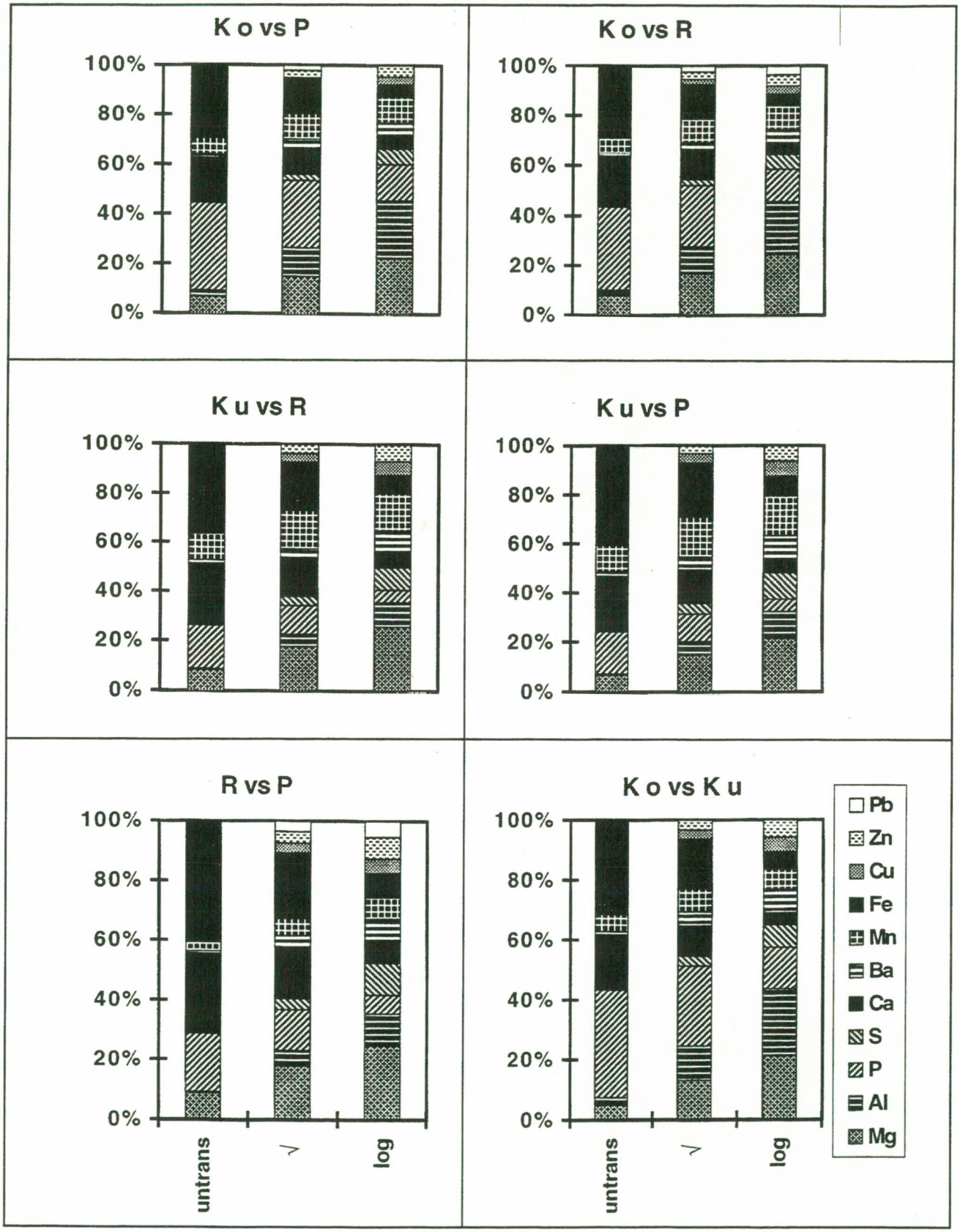

Figure 4. Results of SIMPER analyses showing the effects of transformations on the contribution of individual elements to the dissimilarity between sites. Note the decreased representation of Fe and increased representation of Mg with increasing strength of transformation. Ko, Kedumba opposite; Ku, Kedumba upstream; P, Pocket Creek; R, Ripple Creek. Transformation categories on the X axis are: untransformed, square root and $\log_e (x+1)$.

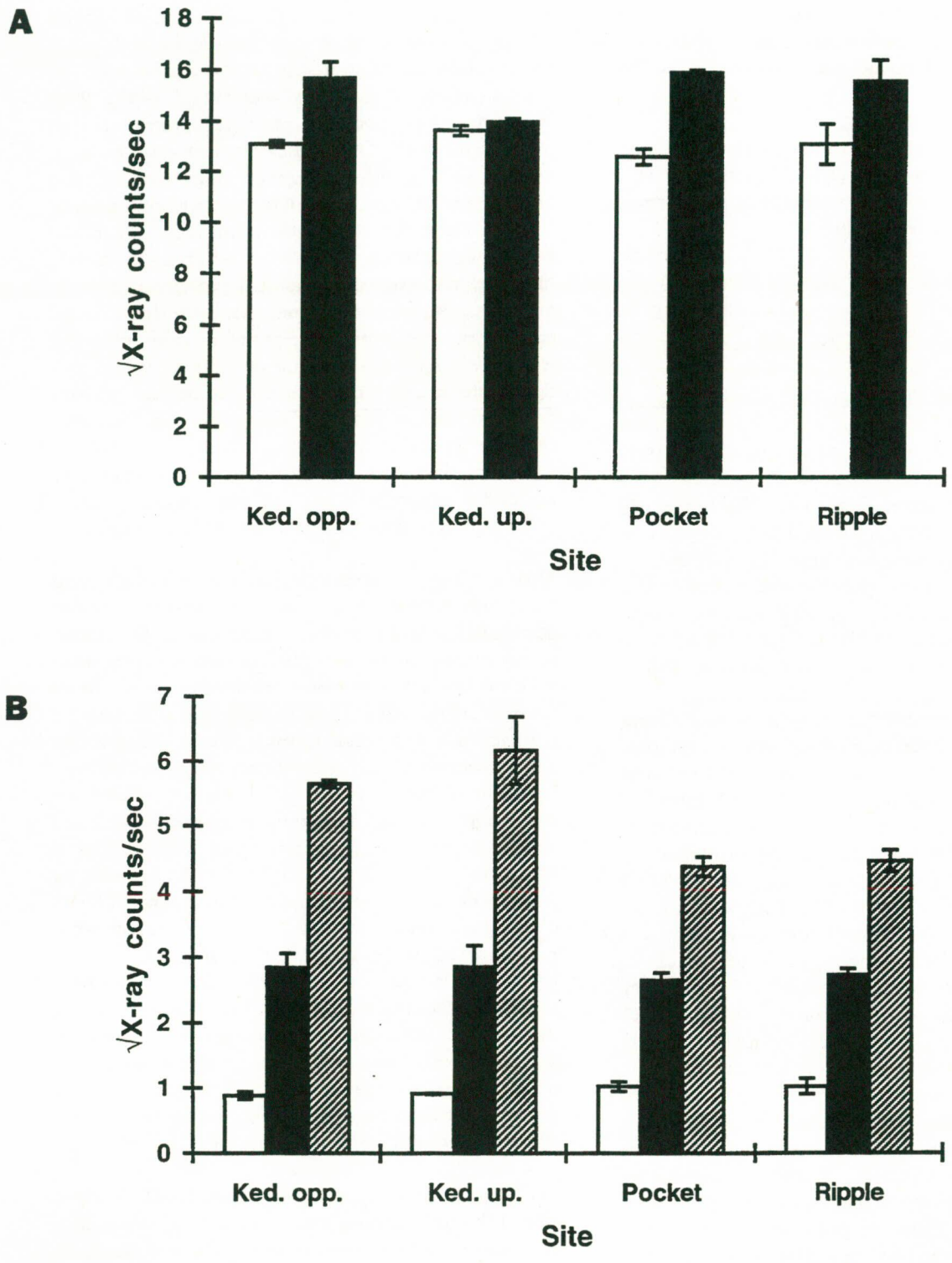

Figure 5. *Means (+/- SEM) for square root transformed X-ray counts/second recorded for 20 granules from each of 3 animals from each site. A. Counts for Ca (open bars) and Fe (solid bars). B. Counts for Mg (open bars), Ba (solid bars), Mn (hatched bars).*

or lithological differences between sites may be best investigated with square root transformation of the data, whereas trace metal investigations may require log transformation.

Bar graphs of the element data indicate that Kedumba upstream mussels had lower Fe and higher Mn levels in their granules compared with the mussels from Pocket and Ripple Creek (Figure 5).

DISCUSSION

Microanalysis of granules in freeze substituted tissue is a new approach to biomonitoring with freshwater mussels and our results indicate that it has promise as a tool to assess the elemental status of the aquatic environment. Other studies of freshwater mussels emphasise the importance of the granules in bioaccumulation (Jeffree *et al.*, 1993; Hickey *et al.*, 1995) and with the techniques used here, they can be analysed directly. Working with granules in sections of freeze substituted tissue avoids the leaching of metals that may have occurred in previous studies where the granules were isolated by chemical extraction before analysis (Pynnönen *et al.*, 1987; Silverman *et al.*, 1987).

The application of freshwater mussels for monitoring metal pollution has been highlighted in several studies where accumulated body burdens were documented (Kraak *et al.*, 1991; Jeffree *et al.*, 1993; Hickey *et al.*, 1995). Like the international 'mussel watch' approach, these studies use homogenates of large numbers of mussels to reduce the influence of inter-individual variability (O'Connor, 1996). In freshwater mussel studies, samples of 20-100 specimens have been used (Kraak *et al.*, 1991; Jeffree *et al.*, 1993; Hickey *et al.*, 1995). The decline in the numbers of freshwater mussels in Australia and elsewhere (Naimo, 1995) however, precludes the general adoption of this approach due to conservation concerns. Our microanalysis study indicates that a small number (n=5-6) of *H. depressa* may be sufficient for use with the granules as the source of data to determine differences among sites using ANOSIM. Moreover, analysis of granules in mantle biopsies may be a useful alternative to whole body analysis for populations in decline because this tissue can be sampled without destroying *H. depressa*. Specimens used in biopsy trials did not exhibit any deleterious effects (Byrne pers. obs.), similar to that found in a recent study of unionid species (Berg *et al.*, 1995).

Metal concentrations in the tissues of freshwater mussels are considered to be largely influenced by the metal content of the granules (Jeffree *et al.*, 1993; Hickey *et al.*, 1995). In whole-body homogenate analyses, regressions against Ca concentration explain a large portion of the among sample variability and the granules are suggested to be the major source of the Ca (Jeffree *et al.*, 1993; Hickey *et al.*, 1995). With the granule X-ray microanalysis data however, analysis of covariance (ANCOVA) indicated that Ca was no more useful than P, Fe or total element count to explain the among sample variability (Byrne and Vesk, unpub. results). These contrasting results undoubtedly reflect the different techniques and body components involved with whole body and individual granule analysis. Whole body analysis includes a large proportion of digestive and gonadal tissue which, in *H. depressa*, have a low granule content compared with that of the mantle. In this study the mantle granules are considered as potential bioindicators in their own right irrespective of their contribution to whole-organism element loads. It would be interesting to undertake a parallel study to compare the element composition of granules and whole body homogenates.

Although X-ray microanalysis has a major advantage in that it allows for direct access to a major site of element accumulation in freshwater mussels, it has limitations in quantitation and in detection of elements present at very low levels. Use of resin-embedded tissue precludes quantitation with this technique due to the contribution of the resin to background. This was not a major problem with granule microanalysis however, because relative elemental levels are useful for comparative studies using consistent preparatory and analytical techniques. Some important ecotoxicological elements such as Cu, Cd, Pb, Zn, Cr and Hg may be present in concentrations below the detection limits of X-ray microanalysis. Most of these elements were not detected in the granules of *H. depressa* and are unlikely to be present in mussels from a relatively uncontaminated location such as Lake Burragorang. We did however, obtain reliable peaks for Pb and Zn from a number of mussels, notably from the Kedumba upstream site and expect that a full range of elements of interest would be detected in mussels resident in, or translocated to, impacted sites.

The X-ray microanalysis data obtained for *H. depressa* from Lake Burragorang will be used as a reference to compare with the elements contained in granules from mussels elsewhere in the Hawkesbury-Nepean system and from mussels translocated to polluted sites (Julli and Byrne, unpub. results). Our preliminary analyses indicate a separation of the Kedumba sites from Ripple and Pocket Creek. This may reflect differences in the trophic status of these sites and may also be influenced by the differences in mussel size. The *H. depressa* at Kedumba are larger than the mussels from Ripple and Pocket. The variation found among the four sites

demonstrates the need for multiple 'reference' sites in biomonitoring studies.

Microanalysis of granular concretions in the tissues of *H. depressa* has potential for use as a comprehensive indicator of a range of metal pollutants, especially in conjunction with multivariate analysis. Multivariate analyses are particularly useful for determining the relationships among samples with complex elemental composition where univariate methods may be limited in scope and sensitivity. This is likely to be true for many X-ray microanalytical studies and for analytical chemical data from ecological studies in general.

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An Ultrastructural and Microanalytical Study of Metal-ion Content in Granular Concretions of the Freshwater Mussel *Hyridella depressa*

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Abstract—Intracellular and extracellular granules in the gonad and mantle of the Australian freshwater mussel *Hyridella depressa* were examined and analysed using SEM, TEM and STEM techniques. As is characteristic of freshwater mussels, this species has extensive aggregations of granular concretions in the extracellular matrix. Energy Dispersive Spectroscopy (EDS) was used to characterise the metal-ion content of these granules and three X-ray microanalytical techniques were investigated to determine the most efficient method of analysis. The granule types were found to differ in their metal-ion composition and concentration, size and degree of annulation with the most abundant granule type having a high calcium phosphate content. Secondary Ion Mass Spectroscopy (SIMS) was also investigated as a comparative technique to characterise the metal-ion profile of these granules, but was found to have limitations in imaging and resolution. The potential for using granular concretions in *H. Depressa* as a method for detecting aquatic toxicants was established. © 1997 Elsevier Science Ltd

Key words: XRMA, SIMS, granules, freshwater mussels, Hawkesbury–Nepean river.

INTRODUCTION

Most of the major macroinvertebrate phyla possess discrete metal-rich granules. These granules are not confined to specific tissues and organs, may be intra- or extra-cellular and are frequently spherical with an internal concentric annulated structure (Morgan, 1984). The most common type of granules found throughout the tissues of freshwater mussels are the calcium phosphate concretions (Brown, 1982; Davis *et al.*, 1982; Pynnonen *et al.*, 1987; Silverman, 1989), known to sequester metal ions. In this investigation the granular concretions in the tissues of *Hyridella depressa* were examined to assess their utility as a biomonitor.

Freshwater mussels are known to accumulate metals from their environment in their tissues, and considerable interest has been generated in the use of these animals as a tool to monitor aquatic systems (Jones and Walker, 1979; Millington and Walker, 1983; Humphrey *et al.*, 1990; Phillips and Rainbow, 1994). The species *H. depressa* is found in both river and lake regions in the Hawkesbury–Nepean system in New South Wales, Australia. Although it has been used in laboratory studies of aquatic pollution (Jeffree *et al.*, 1993, 1995; Markich and Jeffree, 1994), the morphology of this bivalve has been poorly documented to date (Humphrey and Simpson, 1985). This study specifically identifies the granular concretions found in the gonad and mantle of *H. depressa* and characterises their metal-ion content. The most efficient EDS technique for analysing many granules was established and employed to determine the profiles of eleven elements: calcium, phosphorus, iron, copper, barium, magnesium, zinc, lead, sulphur, aluminium and manganese.

A pilot study using a Cameca IMS 5f Secondary Ion Mass Spectrometer, owned and operated by the Australian Nuclear Science and Technology Organisation (ANSTO), was conducted at Lucas Heights, New South Wales to determine the capabilities of this new facility using biological tissue. The objective of this study was to localise, analyse and quantify the metal content of granular concretions in the tissues of the freshwater mussel *H. depressa*. It was anticipated that the results produced with SIMS could be compared to those produced with standard X-ray microanalytical techniques. The ANSTO Secondary Ion Mass Spectrometer is a UHV high mass resolution, high transmission SIMS with direct ion imaging and ion microprobe analysis capabilities. The instrument is equipped with a Duoplasmatron source, producing O_2^+, O^-, and Ar^+ with accelerating voltages from 5 to 17.5 keV, a beam diameter of 0.2–200 μm and a caesium microbeam source with a beam diameter of 0.15–200 μm.

Secondary Ion Mass Spectroscopy (SIMS), developed in the early sixties by Castaing and Slodzian (1962) as a major technique for analysing elemental and mass microstructure in the surface layers of materials, has had limited biological application due to difficulties with the preparation of the biological tissue for the preservation of diffusible elements (Galle, 1982; Lodding, 1983; Burns, 1988; Chandra and Morrison, 1988). Frozen-hydrated (Chandra *et al.*, 1986), Frozen-freeze-dried (Chandra and Ausserer, 1992; Chandra and Morrison, 1992) and Freeze substitution (Zierold and Steinbrecht,

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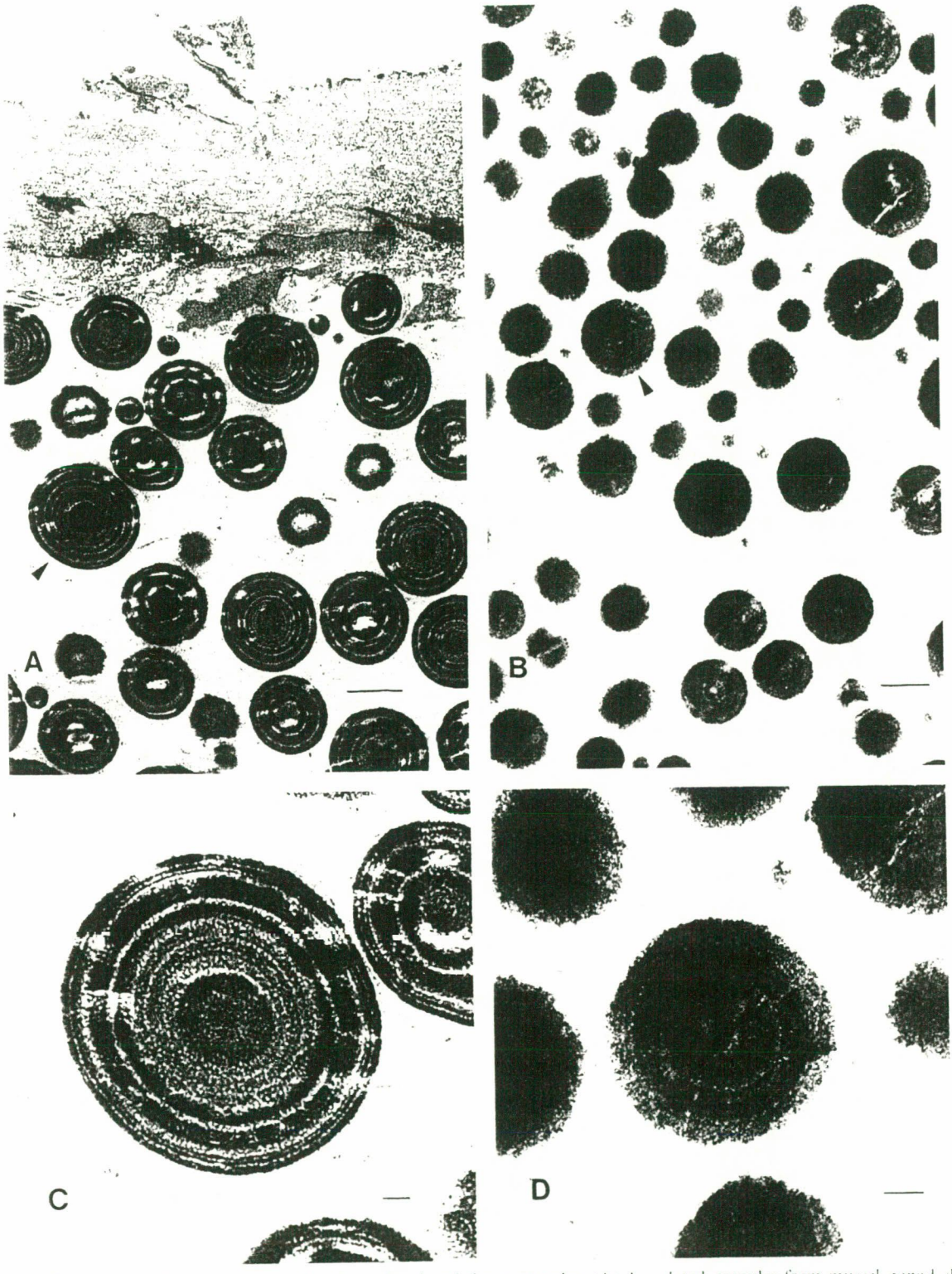


Fig. 1. Transmission electron micrographs of annulated and dense granules. (A) Annulated granules from mussel gonad show clearly defined bands of light and dark material. (B) Dense granules from mussel mantle appear amorphous. Annulations are not present at this magnification. (C) Detail of an annulated granule from (A) (arrowhead). (D) Detail of a dense granule from (B) (arrowhead). At high magnification annulations can be seen. Scale bars: (A)=1 μm, (B)=0.5 μm, (C)=0.2 μm, (D)=0.1 μm.

1987; Hallegot *et al.*, 1990) are the tissue preparation techniques of choice for elemental distribution of diffusible ions. Analysis using frozen hydrated tissue, however, requires a cold stage to be attached to the SIMS microscope and this facility was not available at ANSTO; therefore chemical fixation was utilised in this study. (Chassard-Bouchaud *et al.*, 1992; Mentre, 1992; Mentre and Escaig, 1992).

MATERIALS AND METHODS

Hyridella depressa were collected from the banks of the Nepean River upstream of Douglas Park bridge in June 1994 and from Pocket Creek upstream of the Warragamba Dam in Lake Burragorang in March 1995. Five male mussels from each site were utilised.

For Transmission Electron Microscopy (TEM), gonad and mantle tissue was dissected and fixed in 2.5% glutaraldehyde in 0.02 M Hepes, post fixed in 2% OsO_4 in 0.01 M Hepes, dehydrated through graded ethanols, transferred to 100% acetone and embedded in Spurr's resin. Ultra thin sections, 80 nm, were collected onto nickel 200 square mesh grids and counterstained in 4% aqueous uranyl acetate for 45 min and lead citrate for 15 min. Sections were viewed in a Jeol JEM 1010 electron microscope operating at 80 keV.

For Scanning Electron Microscopy (SEM), the mantle was fixed as above, dehydrated to 100%, critical point dried, mounted onto aluminium stubs and then sputter coated to 20 nm, either with platinum and viewed on a Jeol 35C at 15 keV, or with gold and viewed on the high resolution Jeol 6000 F at 10 keV.

For X-Ray Microanalysis (XRMA), the tissue was prepared as for TEM, but sections were not counterstained. Energy Dispersive Spectroscopy (EDS) analyses were carried out using a Philips CM12 transmission electron microscope operating at 120 keV fitted with an energy dispersive X-ray detection system (EDAX 9900), in both nanoprobe (highly focused beam) and scanning transmission electron microscope (STEM) modes. Three methods of X-ray microanalysis were used viz: (1) linescan, (2) point analysis and (3) area analysis. For linescan analysis, annulated granules from ultrathin sections (95 nm) were analysed by a linescan of 256 points using a spot size of 25 nm, and a STEM magnification of 20000 ×. A scan time of 120 s was used for analysis from the granule edge to its centre. In point analysis sections (95 nm), the light and dark annulations of a granule were probed separately with a spot size of 15 nm and STEM magnification of 100000 × for 100 s. For area analysis, sections of 0.2 μm thickness were collected onto uncoated nickel 200 mesh grids or onto Parlodion coated nickel hole grids and carbon coated. Whole granules were analysed at a STEM magnification of 50000 × for 100 s using fixed 0.4 × 0.4 μm raster windows.

Five granules from four different groups of granules from the same animal for each site were analysed with this method.

For Secondary Ion Mass Spectroscopy (SIMS), the mantle tissue was embedded in paraffin wax or Spurr's resin. For resin embedment, the mantle was dissected and fixed as for TEM processing. Sections (1 μm) were floated onto a water bath and placed flat onto four different specimen holders: (1) uncoated nickel 200 μm meshed grids, (2) aluminium stubs 20 mm diameter, (3) aluminium disks 20 mm diameter or (4) pure gold slides 5 × 5 × 0.05 mm attached on top of 40 mm aluminium disks with double sided tape at the corners; the edges of which were coated with silver conductivity paint. All four specimen support systems were gold sputtered to 5 nm.

Mantle tissue from the same specimen was prepared in parallel for wax embedment. For this study the tissue was fixed in 3% gluteraldehyde in 0.02 M Hepes pH 7.2 for 6 hr. Tissue was rinsed in 0.1 M phosphate buffer at pH 7.2, dehydrated in graded ethanols and embedded in paraffin wax. Sections (5 μm), were collected dry, laid flat and trimmed to fit on the 5 × 5 × 0.05 mm gold slides. Distilled water containing horse serum was dropped onto the gold slides and the trimmed wax sections were floated out onto the slides, dried on a hot plate, 40°C, dewaxed by dropping Histoclear onto the slide and gold coated to 5 nm.

Using the ion microprobe, a mass spectrum was obtained for each specimen using a primary O_2^- beam and operating with full transmission, i.e. no apertures used, at 12.5 keV, a primary current of 5.0 nA and spot

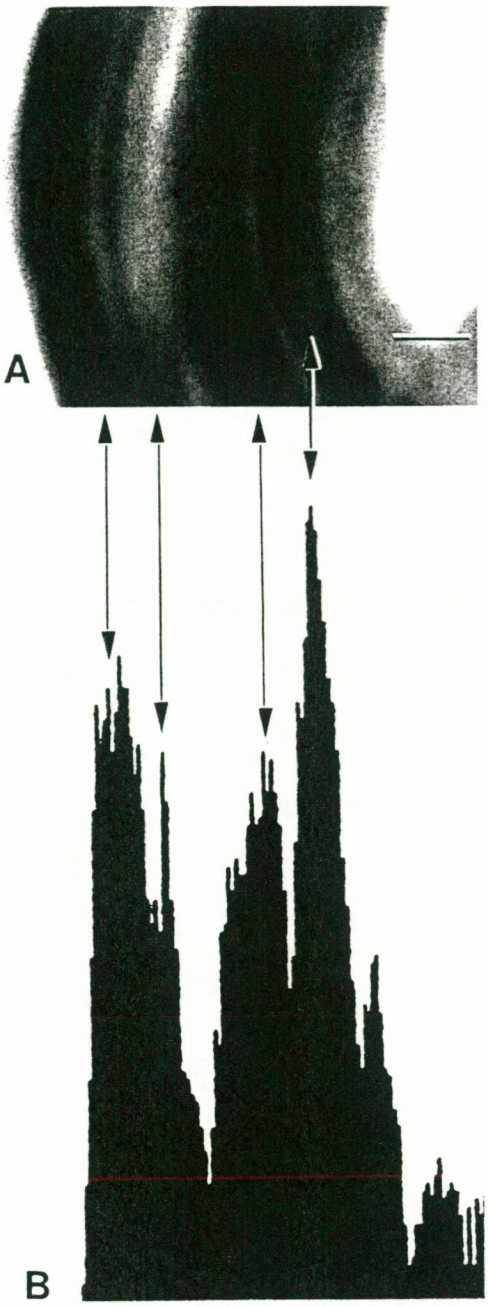


Fig. 2. EDS linescan of an annulated granule from the edge to the centre, (A) at a working STEM magnification of 200000 × the concentric bands of light and dark material display subannulations using phosphorus as an example. (B) The peaks of the scan correspond to the dark bands (arrows) and the troughs correspond to the light bands. Fine peaks correspond to the subannulations. Scale bar: (A)=14 nm.

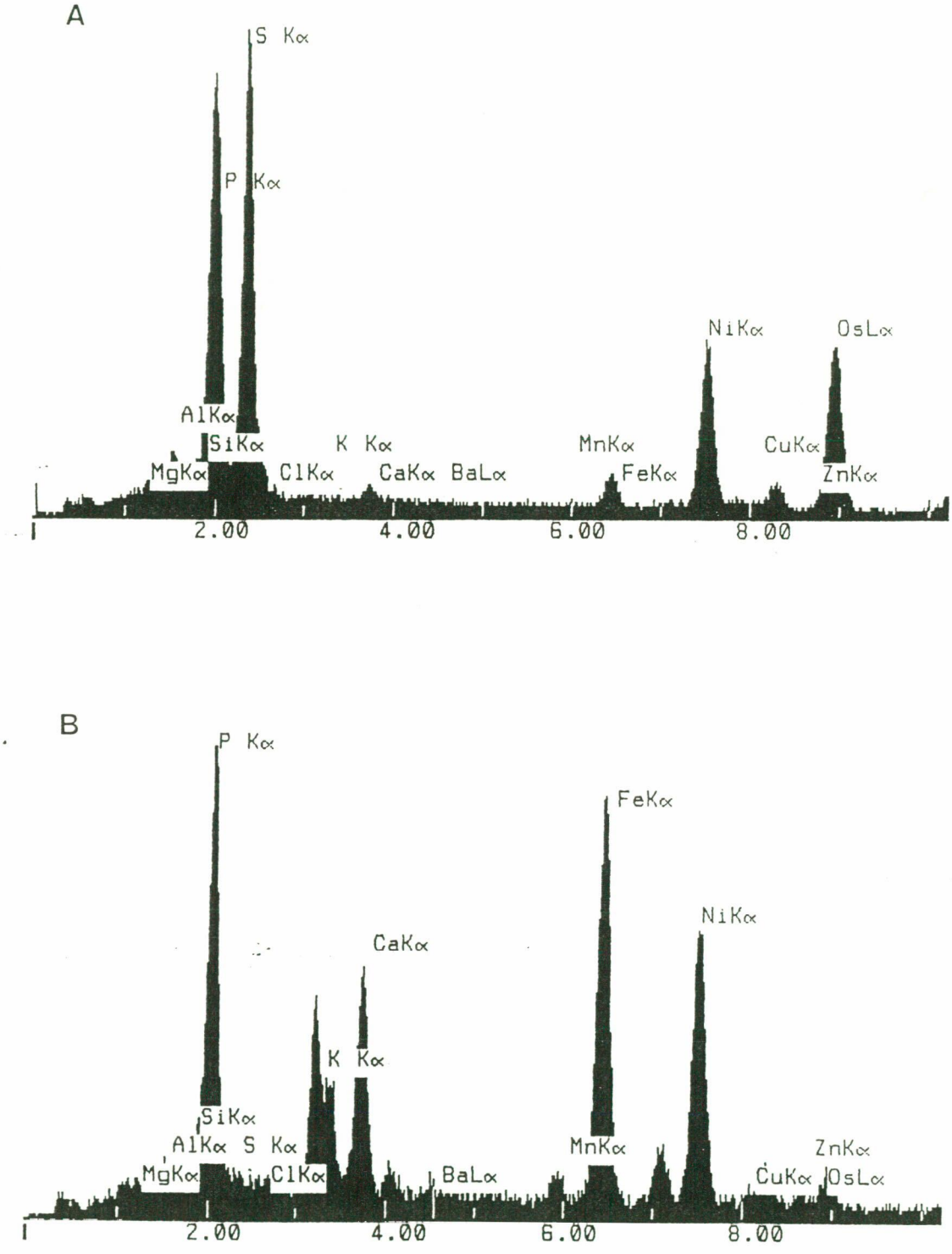


Fig. 3. A–B.

Fig. 3. EDS spectra comparing two different granules from the same animal. (A) Spectrum showing very low levels of iron and calcium and very high levels of sulphur. (B) Spectrum showing high phosphorus, calcium and iron peaks and no sulphur peak. (C) and (D) Point analysis of annulated granule. (C) EDS spectrum of light annulation. (D) EDS spectrum of dark annulation. Note: metal composition appeared to be the same for both light and dark annulations.

size 15 μm using: (1) a 50 μm raster and (2) a 250 μm raster. Images were also obtained using the ion microprobe operating at 15 keV with a primary current of 4.0 nA for (1) calcium, phosphorous and iron using a 500 μm raster and spot size 15 μm, and (2) for calcium, phosphorus, iron and magnesium using three rasters, 90 μm, 168 μm and 190 μm with spot size 10 μm.

RESULTS

Morphology of granules

Granules were located both intra- and extra-cellularly and varied considerably in their structure from the highly annulated type (Fig. 1A and C) to a dense type

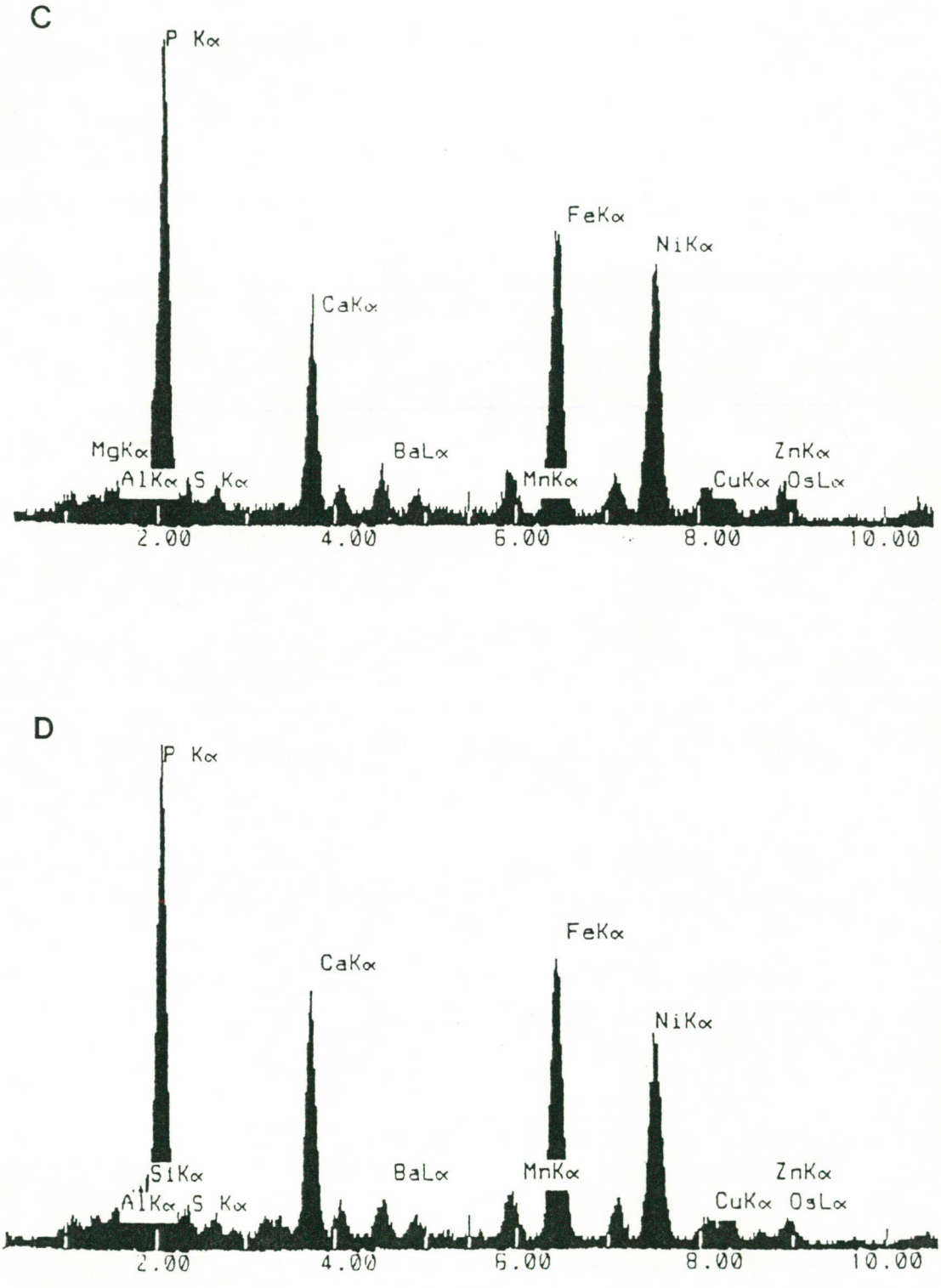


Fig. 3 C–D.

(Fig. 1B). A study of the highly annulated granules revealed distinct concentric bands of light and dark material at a working magnification of 70000× (Fig. 1C). However, at a working magnification of 200000×, the annulations were seen to be comprised of further sub-annulations which were not as clearly defined (Fig. 2A). In comparison, the dense granules appeared uniform, amorphous and lacked obvious annulation (Fig. 1B). However, examination of these granules at a working magnification of 70000× revealed that they also exhibited some annulation (Fig. 1D).

Intracellular granules were found either singly or in small groups and ranged in diameter from 0.1 μm to 2.0 μm. X-ray microanalysts of one type of intracellular granule, referred to as "soft" granules, revealed they had a different metal composition compared to "dense" granules. EDS analysis of these "soft" granules (Fig. 3A) showed low concentrations of iron and calcium and

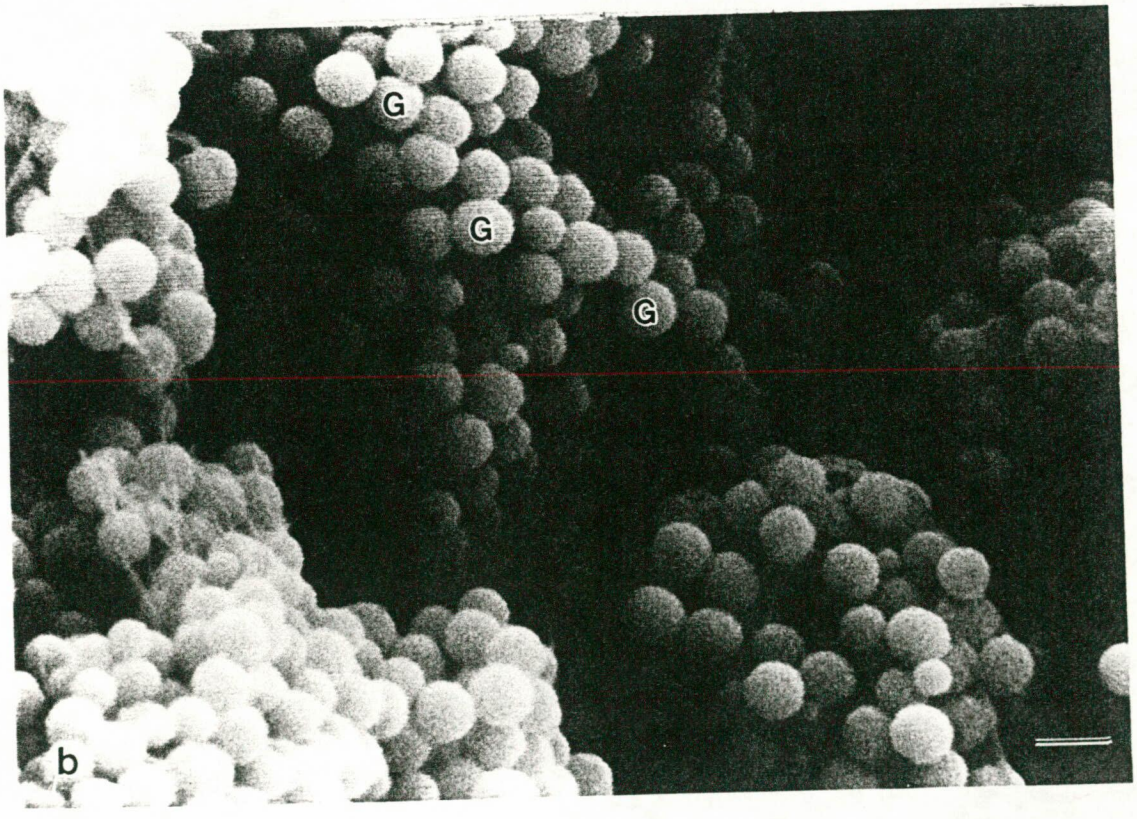
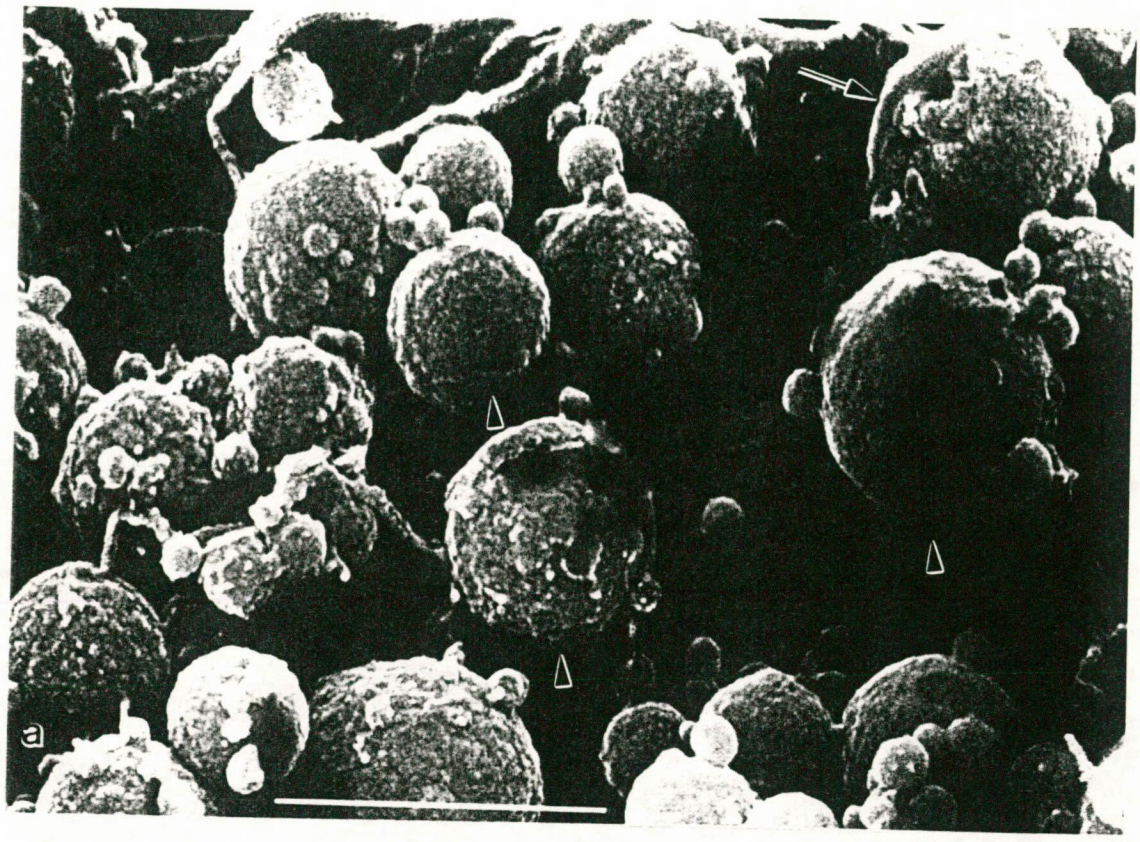


Fig. 4. SEM micrographs of mussel mantle granules. (A) Transverse section mantle. Groups of granules—arrowheads. Scale bar=6.7 μm. (B) Higher magnification of groups of granules from (A). G=individual granule. Scale bar 0.8 μm. (C) Individual granules (arrowheads). Higher magnification of (D). Scale bar=1.0 μm. Note: arrow points to granule damage during processing and reveals evidence of lamellation. Gold sputtered. (D) Group of granules in mantle. Scale bar 1.0 μm.

high concentrations of sulphur and phosphorus when compared with EDS spectra obtained from dense granules (Fig. 3B).

Extracellular granules in the gonad were uniquely annulated (Fig. 1A and C) and were larger in diameter (2.5 μm), than extracellular annulated granules in the



mantle which varied between 0.5 and 1.5 μm in diameter.

The most abundant type of granule was the extracellular "dense" granule. These granules were found in large groups scattered throughout the mantle (Fig. 4A). Within groups, the granules were of a similar size but this size varied between groups. Scanning electron micrographs showed the granules to be spherical (Fig. 4B and C) and to be associated in groups (Fig. 4D) which were irregular in shape. The average diameter of the granules was 0.5–0.8 μm.

X-RAY MICROANALYSIS

Linescan analysis

Linescan analysis through an annulated granule from the outer edge to the centre demonstrated the differing concentrations of metals present in each band (Fig. 2A and B). The light and dark bands were clearly represented by differences in metal peaks. Finer annulations were also demonstrated by individual peaks. Due to the inherent overlap within a continuous line scan, interpretation of these peaks is difficult.

Point analysis

In contrast to line analysis, point analysis (Fig. 3C and D) of the light and dark bands indicated that both bands consisted of the same metals. This suggested that the difference in the bands was one of concentration rather than composition.

Area analysis

Area analysis provided the benefit of an overall assessment of the metal levels in individual granules. Fig. 5A demonstrates the fixed $0.4 \times 0.4\,\mu$m raster window that was placed randomly over the granule for area analysis. The EDS spectrum obtained from this method of analysis is represented in Fig. 3. Statistical analyses performed on the calcium phosphate granules employed ratios to calcium as the values for this element were less variable when compared with phosphorus. When the elemental values for each of the twenty granules per animal were plotted as histograms (Fig. 5B and C), minimal variability of metal-ion content between granules of the same animal was demonstrated.

Specimens placed on Parlodian-coated, nickel hole grids gave the same results as uncoated nickel 200 square mesh grids, but the latter were less fragile to work with and required less time with their preparation.

SIMS

Ion microprobe image

The images obtained from both resin and wax sections demonstrated the effects of variability of tissue thickness—either from uneven cutting or the tissue not lying flat on the grid. This variability of topography affected the sputter yield of element concentration present and was particularly apparent with paraffin wax embedded samples. This variability of topography was observed with all specimens and resulted in aberration of images such as blurring and stretching, etc., and which may have occurred due to the differing heights of granules in the specimen. Identification of these erratic, non spherical images as groups of granules was inconclusive.

Element distribution of four elements was analysed, i.e. calcium, magnesium, iron and phosphorus. Although comparison of the results from the images obtained for each raster, i.e. 90, 168 and 190 μm rasters using a 10 μm spot and a 500 μm raster using a 15 μm spot size, demonstrated the higher resolution obtainable with a smaller spot size and raster; it was difficult to determine from these results if the particles being scanned were the calcium phosphate granules of interest.

Mass spectra

A mass spectrum is represented by a line bar graph for isotopes of increasing atomic mass and allows the presence and abundance of each isotope to be interpreted. However, only low resolution mass spectra could be obtained using (1) a 50 μm raster and (2) a 250 μm raster. Information obtained from these mass spectra is difficult to interpret as all possible variations of polyatomic ions at a given mass are present, e.g. Fe and CaO both have an isotope at atomic mass 56. High mass resolution spectra would need to be obtained in order to separate these peaks. It was not possible with the equipment available to obtain high mass resolution spectra while working with a spot size of 15 μm.

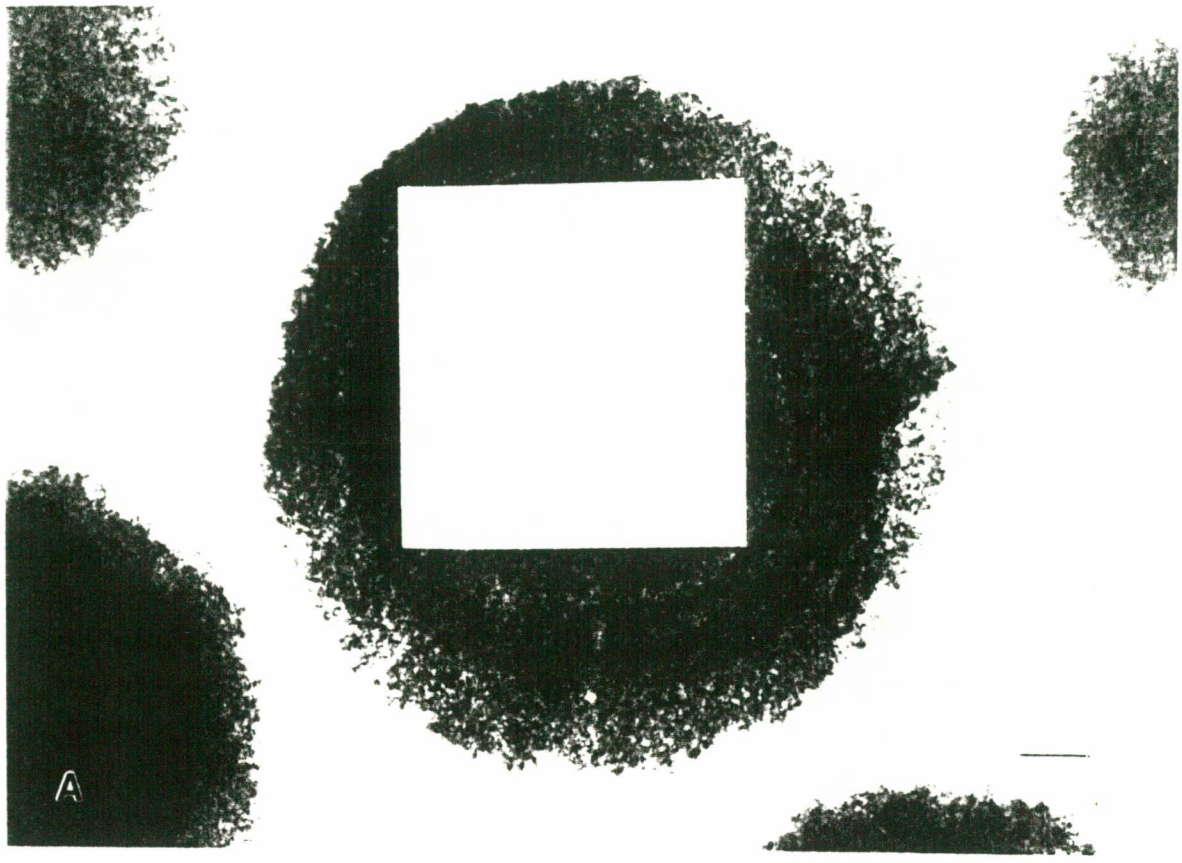
DISCUSSION

Morphology of granules

Different types of calcium concretions are found throughout the tissues of bivalves including the mantle (Istin and Girard, 1970; Simkiss, 1976; George, 1982; Watabe and Kingsley, 1989). This ultrastructural study demonstrated several different types of granules present in the tissues of *H. depressa*. It appeared that all granules exhibited some annulation. Extensive examination of the dense granules indicated that magnification was critical to the visualization of the annulations within these granules (Fig. 1D).

X-ray microanalysis

Assessment of the methods used for the analyses of metal content in the granules indicated that both linescan and point analysis had inherent problems in determination of metals at specific sites within the granule. With linescan analysis, probe overlap caused difficulty in the definition of individual bands within the granule. This problem was somewhat overcome by the smaller discretely placed probe used with point analysis. If a particular annulation is of interest for bioaccumulation studies, then point analysis would be the method of preference. However, an increase in the working magnification results in a loss of definition (Fig. 2A), and thus the fine subannulations within the granule would not be



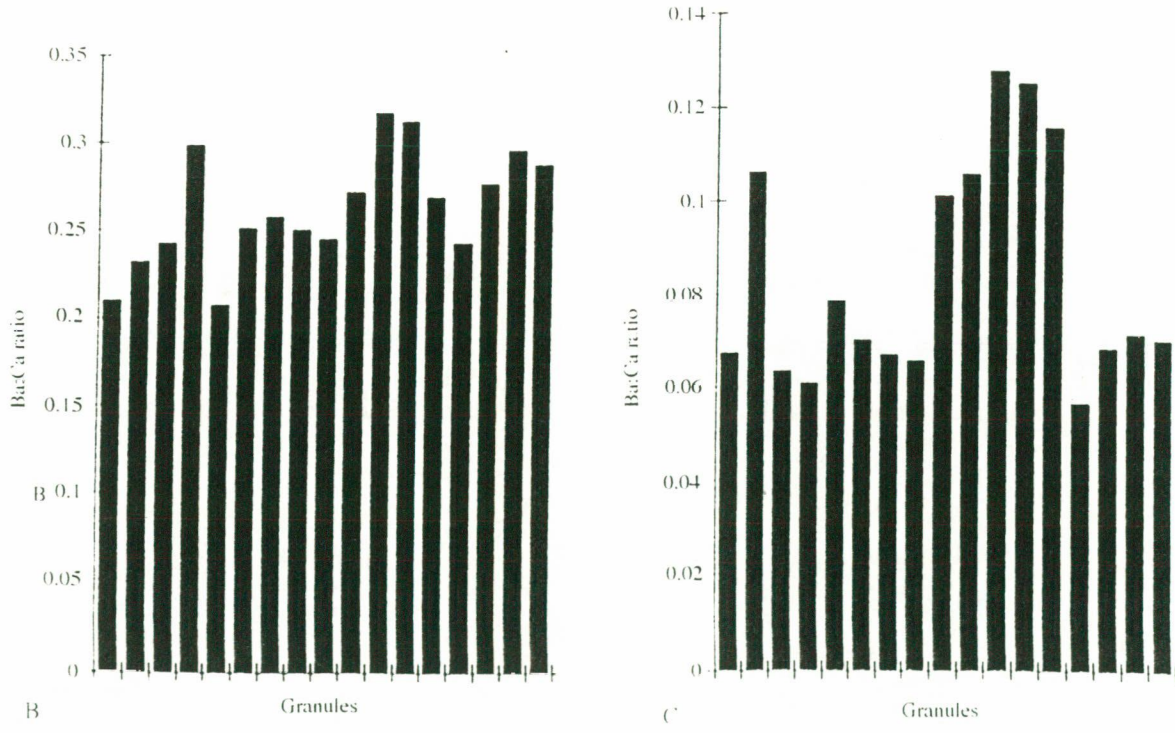


Fig. 5. (A) Area analysis of a dense granule. A typical granule of average size is used to demonstrate the $0.4 \times 0.4\ \mu m$ raster window that is placed randomly over the granule for area analysis. Scale bar=0.15 μm. Note: at high magnification an annular arrangement is observed. (B) and (C) Metal ratios for barium calculated for individual granules $n=17$, for an individual mussel. (B) Barium ratioed to calcium. (C) barium ratioed to phosphorous. Between granules of the same animal calcium distribution appeared to have less variability.

sufficiently differentiated and the data obtained not discrete.

Although the line scan and point analysis methods were useful for obtaining the metal profiles of individual annulations, they were not efficient methods with which to obtain a large sample size for statistical analysis. Area analysis, however, allowed for a comparatively rapid analysis of many granules. Due to the fixed window of analysis, which encompassed the majority of a granule, the resultant profiles were representative of overall granule metal content. Moreover, calculation of the metal ratios for each granule from the area analyses would allow for statistical determination of metal levels in granules from different tissues, animals, sites, etc.

The fact that some granules were found to be rich in S and P with only trace amounts of Fe and Ca concurs with the granular content disparity reported by Marshall and Talbot (1979), Mason and Simkiss (1982), Mason *et al.* (1984), Viarengo (1989) and Marigomez *et al.* (1990). However, although the metal-ion composition was similar between the light and dark annulations, Marigomez *et al.* (1990) suggested that the elemental composition between annulations of the same granule in an estuarine winkle may differ.

The results of this study indicated that area analysis of individual granules is the most useful method for metal determination by EDS. It is anticipated that this method will become an important tool for the use of granular concretions in freshwater mussels as bioindicators with which to monitor aquatic systems.

SIMS

The smallest working spot size used, which minimized charging or which did not destroy the sample, was found to be 10 μm. However, images produced from this large spot size resulted in low resolution and therefore the granules could not be visualised. The inability to select specific sites within the specimen presented a major problem in that groups of granules could not be identified. To raster morphologically unknown areas became time consuming and introduced uncertainty. Certainly it was not possible to locate an individual granule ($\sim$1 μm) and place a probe onto it for analysis.

The results obtained from this pilot study were disappointing since visualisation of desired morphological areas could not be determined. It would be necessary to predetermine if groups of granules were present in the chosen sample by electron microscopy or microprobe analysis, e.g. XRMA. These techniques, in conjunction with SIMS are microanalytical tools that should be used to provide complementary results on chemical contaminants, i.e. morphological and chemical identification (Chassard-Bouchaud *et al.*, 1989, 1992; Prince, pers. comm.).

When a suspected group of granules was imaged, resolution became the major problem resulting from the limitations of the O_2^+ primary ion source available with the ANSTO SIMS. Other laboratories outside Australia have overcome this problem by utilising a gallium primary ion source (Chassard-Bouchaud, 1988; Levi-Setti *et al.*, 1988). This instrument allows demonstration of topographic and elemental imaging with lateral resolution attaining 20 nm. Gallium sources produce a denser, more finely focused primary beam than O_2^+ primary beams and this improves the secondary ion transmission. This allows the specimen to be readily imaged as the smaller primary beam results in better resolution. ANSTO SIMS does not have a gallium source and imaging performed with an O_2^+ primary beam results in lateral resolutions of 5–15 μm. Also, although it may be possible to visualise the groups of granules, it was not possible to probe individual granular concretions approximately 1 μm in diameter.

The SIMS method of analysis, when operating at high mass resolution, makes it possible to obtain a total analysis of the elemental composition of the tissue. However, the elements detected in this study were in low concentration and their detection required an increase in beam current with resulting sample destruction. To prevent this damage, only low mass spectra were obtained and ion microscope images could not be visualised.

Specimen preparation for SIMS required special attention. Irregular topography resulted in charging of the specimen and produced aberrations in the ion image. Mass spectra, therefore, became difficult to interpret due to interferences between primary and secondary ions.

CONCLUSION

The inherent charging effects of biological tissue makes SIMS analysis of insulators difficult (Linton and Goldsmith, 1992). Current limitations, especially in comparison to X-ray microanalysis, centre on lateral spatial resolution and imaging. XRMA employing EDS has been demonstrated to detect eleven elements Ca, P, Fe, Cu, Mg, Al, Ba, Mn, Zn, Pb and S in the dense granules of *H. depressa*.

Acknowledgements—Our thanks to Dr Prince and ANSTO for providing the SIMS facility and to Drs Ian Kaplin and Clive Knockolds from the Electron Microscope Unit, University of Sydney. This study was partially supported by a grant from the Environmental Trusts.

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MEDIA
Appendix 2

Mussels and heavy metals

Freshwater mussels are being used as bio-indicators of heavy metal levels in Sydney's Hawkesbury-Nepean water system. Using energy dispersive X-ray microanalysis, the metals and other elements are detected in the mussels' extracellular granules.

Maria Byrne and colleagues at the University of Sydney analyse the mantle tissue from specimens taken from four sites in Lake Burragorang. Because the species *Hyridella depressa* numbers are declining, Byrne considers the mantle useful because she can biopsy it without killing the animal. The mantle is also a very thin tissue and amenable to sectioning.

Mantle tissue from each specimen is prepared by acetone freeze-substitution after cryofixation with copper-tipped pliers cooled to liquid nitrogen temperature. Sections of specimens embedded in resin are analysed using a Philips CM12 scanning transmission electron microscope operated at 120kV equipped with an Edax X-ray detector. Inherent problems with X-ray microanalysis include beam variations and section thicknesses differ, so there will always be artefacts, says Byrne. "But if you do a large enough sample size, this will be minimised."

Absolute quantities of elements cannot be determined using this system. "You can only quantify by grinding the whole animal and get the metal levels as a percentage of body weight. But if you consistently use the same method for all samples, the assumption is that the error will also be consistent."

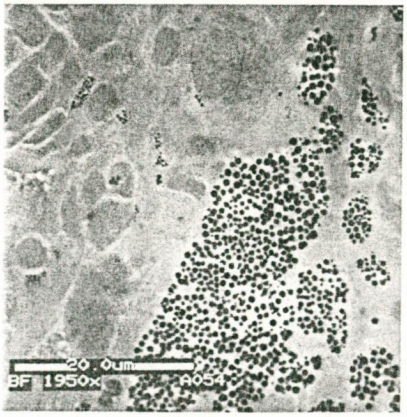
Because freshwater mussels are sedentary suspension feeders, they spend most of their in the one place, providing an indication of the water quality of that site for its lifetime, says Byrne. In this way, they can provide a historical record as well as a current record.

How the mussels store specific elements depends on the element involved and the physiology of the animal. "A lot just sequester and store the metals in their tissue. They can detoxify them and put them into extracellular granules and they are neutral to the animal. For some metals, such as zinc, they can metabolise them and incorporate them into metallothionine proteins, so I won't pick them up. So far I've been able to pick up all the metals in various concentrations including copper, lead, nickel, and zinc. Other trace elements detected include as calcium and phosphorus (a pollutant from detergents, fertilisers, etc)."

The lake where the animals are taken, Lake Burragorang, is the catchment area for Sydney's drinking water. It is not polluted and is used as a reference site. "But even here we are detecting differences in element concentrations. Because of the discrepancies in age and biology of the animal, you can't only use one or two reference sites."

Future work will involve analysis of freshwater mussels that have been taken from Lake Burragorang and placed in polluted areas downstream of the dam.

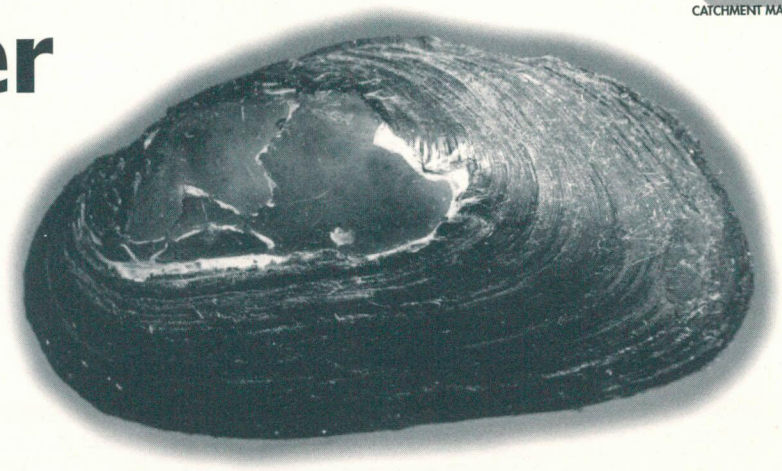
The research is being supported by the NSW government through its environmental research trusts.



Aggregations of calcium phosphate granules in the mantle of the freshwater mussel, detected using an STEM with an X-ray detector.

Hawkesbury Nepean CATCHMENT MANAGEMENT TRUST

Freshwater mussel malaise



Surviving mussel populations in the Hawkesbury-Nepean system are dominated by large adults like this specimen of Hyridella depressa. *The annular rings on the shell show the age of the mussel.*

Freshwater mussels may be on the decline in the Hawkesbury-Nepean river system, similar to their decline in large river systems in North America and Europe, according to Sydney University researcher, Maria Byrne.

Byrne and her research team have found populations of mussels dominated by large adults which indicates that juvenile recruitment has not occurred in these populations for some time.

Byrne's research indicates that the decline of mussels may be due to a reduction of the fish used as a host by the mussel larvae.

Alternatively, the habitat may no longer be suitable for young mussels due to pollution or disturbance. The mussels appear to be particularly sensitive to changes in their habitat.

Byrne and her team have searched the river system for two major genera of freshwater mussels, *Hyridella* and *Velesunio*. They are researching the use of these genera as bio-indicators of heavy metal levels in the river system.

Freshwater mussels provide an indication of water quality of an area over time because they are sedentary suspension feeders and spend all of their life in one area. They are natural biological scrubbers, due to the large volume of water that passes through their body each day. To feed, they create an inhalant current from the surrounding water and basically filter off food particles such as zooplankton and organic detritus.

Different elements picked up by filtering are stored by the mussel in different ways. Byrne's research team uses energy dispersive X-ray micro-analysis to detect levels of metals and other elements in the mussels' extracellular granules.

"A lot of mussels just sequester and store the metals

Mussels are natural biological scrubbers, due to thelarge volume of water passing through their body each day. Photo shows Hyridella depressa *in Lake Burragorang. Photographed by Joe Buttita, Blacktown Council.*

Mussel-search – *how you can help*

Byrne is compiling a map of fresh water mussel populations along the Hawkesbury Nepean River system. You can help by keeping an eye out for these mussels and notifying the Trust.

Freshwater mussels prefer larger river systems as well as lakes and waterholes. They most likely occur in shallow water, on stable sandy, silty or muddy bottoms – never on exposed rocky bottoms or unstable sand and mud. There may also be scratched and opened shells on the edges of waterways, left by water rats and other predators.

If you see any evidence of mussels contact Nikki Plunkett-Cole at the Trust on (045) 77 4243 and we will notify Byrne's research team.

Please don't remove the mussels from their local habitat.

Make sure you have information on river location and access to the area such as walk from... and nearest crossroad etc, as well as the date, mussel appearance, rough size and numbers.

in their tissue. So far I have been able to pick up various concentrations of metals including calcium, phosphorus, iron, copper, lead, zinc and aluminium." says Byrne.

This filtering mechanism of mussels can be used to purify water. For example, the Plumpton Park Wetlands, established by Blacktown Council to treat stormwater, includes freshwater mussels.

Future work for the research team will involve analysis of freshwater mussels that have been taken from Lake Burragorang which is the catchment area for Sydney's drinking water and placed in polluted areas downstream of the dam. This will determine the level of pollution in this area of water.

Know your mussels

The two most common mussels in the Hawkesbury-Nepean are very similar in appearance, distinguished by their somewhat different shell shapes.

Hyridella depressa (maximum size) - note the elongated shell shape (the coin is a 20c.)

Velesunio ambiguus (approaching maximum size) - note the oval shell shape (the coin is a 20c.)

The complex life history of the freshwater mussel

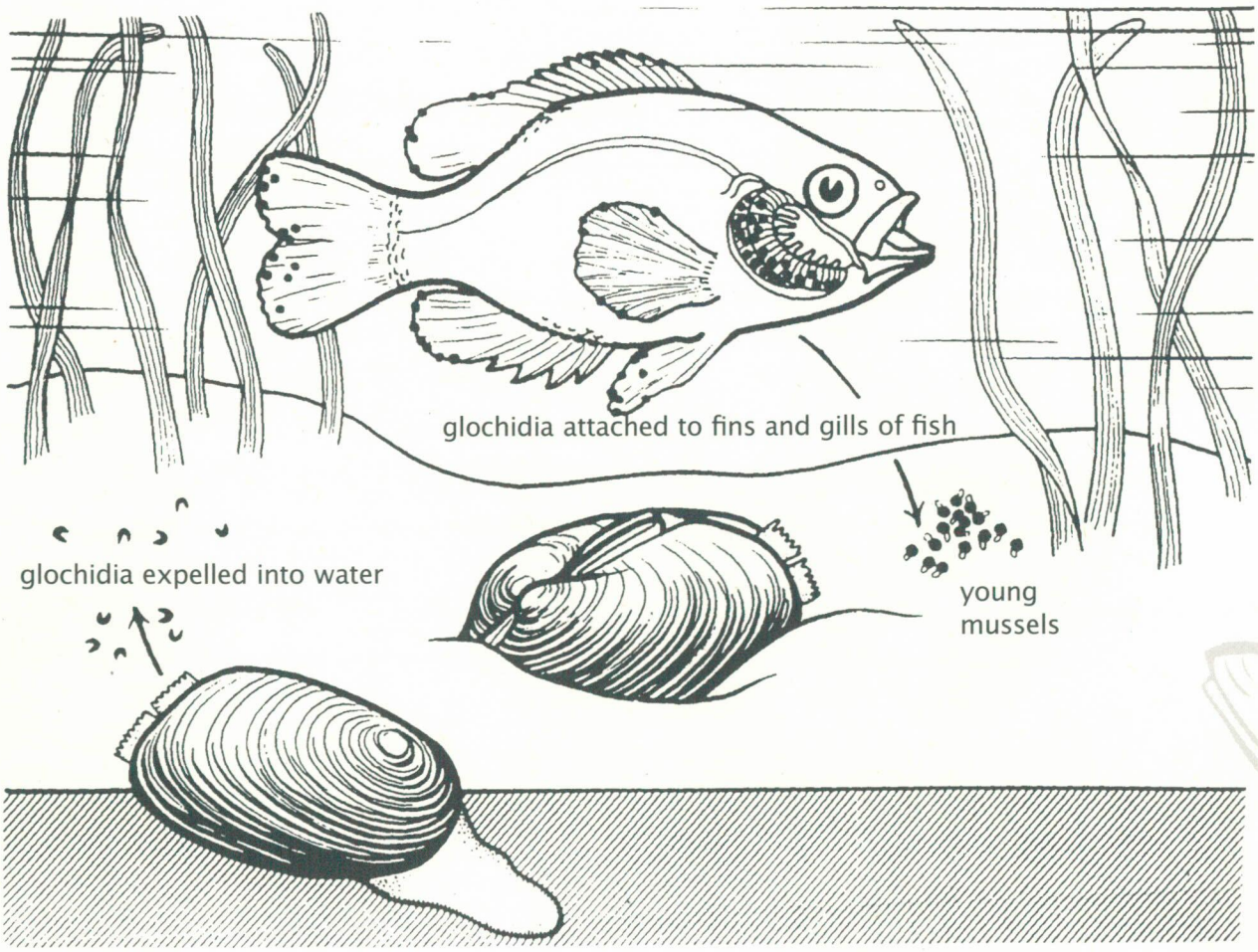


Illustration from Pearse et al (1987)

Northern Herald Thurs Sept 5 1996

Mussel Definition

Monitoring the Hawkesbury's health

Threats to public health and industry continually emerge along the ecologically troubled Hawkesbury-Nepean River, Sydney's aquatic playground. **Col Allison** investigates the latest indicator of toxic pollution – freshwater mussel malaise. Main photograph by **David Hancock.** See story page 4

MUSSEL definition

BY COL ALLISON

DR MARIA Byrne, an underwater Sherlock Holmes of the scientific world, is described by colleagues as "the woman who dives in Sydney's water supply". In fact, thanks to her unique and disturbing study indicating that freshwater mussels are in decline, she is the only outsider to snorkel in Lake Burragorang, the unpolluted and restricted waters behind Warragamba Dam.

The Irish-born woman is a lecturer in anatomy and histology at Sydney University. She dives up and down the Nepean-Hawkesbury River system seeking clues to a biological jigsaw puzzle while investigating the location, health and reproduction of two major genera of freshwater mussels living in shallow water *(Hyridella depressa* and *Velesunio ambiguus).*

And, like the deep waters near the dam walls, the news on mussels – which have been documented at 125 years of age in Germany – is quite gloomy. They appear not to be reproducing as they once did, with wild populations dominated by large adults and containing few or no juveniles – a similar depletion pattern to North America's. (In the United States, a thriving mussel industry existed on the Mississippi River as early as 1916, when 20,000 people were employed in a $12.5 million-a-year conversion of raw mussel shells to "pearl" buttons.)

Dr Byrne and her team of two research assistants and an undergraduate student have found mussels below the dam to be "locally extinct". Yet within living memory they were so thick in their sandy, silty or muddy bottom habitat that they sometimes formed a "carpet" of shells from bank to bank in prime locations, according to anecdotal evidence.

The conclusion? "I suspect the intermediate fish hosts, which play a large part in the complex life history of the mussel, are significantly depleted," Dr Byrne said.

"We haven't found any juvenile mussels in the Hawkesbury, which is worrying."

Dr Byrne said the reduction of the mussel population could signal serious environmental decline.

"Like canaries used as gas-detectors in coal mines, mussels are the natural biological scrubbers, the cleaners of the rivers. They remove toxicants and nutrients from the water and purify and clarify it, helping keep down harmful algal bloom," she said.

"Freshwater animals have not been well studied in Australia, except in the Murray-Darling, but only after the Murray cod had bolted, following the demise of the river system. Mussels can be used for habitat remediation. The Plumpton Park Wetlands, established by Blacktown Council to treat stormwater, uses freshwater mussels to filter and purify the water."

If there is not a major decline in stocks of native fish – which pick up mussel glochidia (seed) via their gills and fins and deposit them later as young mussels – then pollution, or disturbances such as dam-water release, may be the reason behind the disappearance of the Sydney mussel.

The most dramatic extinction of mussels took place in the US shortly after World War II with the building of a massive hydro-electricity scheme in the Tennessee Valley, similar to our Snowy Mountains project. "Lots of species just disappeared," Dr Byrne said.

She added: "Some pollutants at low level act as hormonal disruptors, which affect animal reproduction. We've had animals in cages for nine months – about 720 mussels in four cages at six sites on the river – studying their reproductive response to monitor the river's aquatic health. The absence [of young mussels] indicates a decline in the river system. But the cause and effect of this decline in not yet entirely clear."

The potential seriousness of the mussel malaise is more easily understood when one considers that tests lasting six weeks revealed mussels survived living in pure sewage plant effluent – "no troubles at all," said Dr Byrne.

Part of her work is monitoring mussels as bio-indicators of heavy-metal levels. She will soon begin assessing freshwater mussels taken from the pure waters of Lake Burragorang and placed in polluted waters downstream of the dam.

Since mussels are sedentary suspension feeders – they filter off food particles of zooplankton and organic detritus – they spend most of their life in the one place. As such, they are accurate indicators of water quality in given sites for their lifetime, which can be 30 to 60 years in Sydney. In this way they can provide an historical record and a current one.

A useful attribute of mussels is that they do not have to be routinely killed to be studied. Sections of their mantles can be taken for a biopsy without too much trauma for the animals. Dr Byrne has found that mussels deal with pollutants in different ways – they can detoxify them and store them in tissue.

"Some metals, such as zinc, can be metabolised so I won't detect them. So far I've been able to pick up metals such as iron, copper, lead, nickel and aluminium, in varying concentrations. Other elements detected include calcium and phosphorus, a pollutant from detergents, fertilisers, etc."

A Balmain resident, Dr Byrne, 40, has spent 18 years studying the reproduction and development of animals, mostly marine creatures, around the world.

But she was not depressed by her intermediate findings on mussels. Like other scientists in differing disciplines before her, she is well aware that the fragile ecology of the Nepean-Hawkesbury River is currently at ebb tide. Only last week the threat of toxic dinoflagellate, an exotic micro-organism thought to have been introduced from ship's ballast water, emerged from new research by the Environment Protection Authority. Under the right conditions these organisms could multiply and contaminate filter-feeding animals like mussels, while posing a serious, albeit temporary, threat to the commercial oyster industry.

But she believed the tide would turn, thanks to the demands of community environmental concerns which have seen the evolution of single-controlling agencies like the Hawkesbury-Nepean Catchment Management Trust, and the NSW Environmental Research Trusts that fund her research. After all, it's not all that long ago that 20-odd bodies had a say in what happened along the river.